

The University of Maine
DigitalCommons@UMaine

Electronic Theses and Dissertations

Fogler Library

2005

Endocrine Disruption in Atlantic Salmon (*Salmo salar*) Exposed to Pesticides

Benjamin W. Spaulding

Follow this and additional works at: <http://digitalcommons.library.umaine.edu/etd>



Part of the [Aquaculture and Fisheries Commons](#), and the [Zoology Commons](#)

Recommended Citation

Spaulding, Benjamin W., "Endocrine Disruption in Atlantic Salmon (*Salmo salar*) Exposed to Pesticides" (2005). *Electronic Theses and Dissertations*. 351.

<http://digitalcommons.library.umaine.edu/etd/351>

This Open-Access Thesis is brought to you for free and open access by DigitalCommons@UMaine. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of DigitalCommons@UMaine.

ENDOCRINE DISRUPTION IN ATLANTIC SALMON (*Salmo salar*) EXPOSED
TO PESTICIDES

By

Benjamin W. Spaulding

B.S. University of Maine, 1997

A THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
(in Zoology)

The Graduate School

The University of Maine

May, 2005

Advisory Committee:

Terry Haines, Professor of Zoology, Advisor

Rebecca Holberton, Associate Professor of Biological Sciences

Rebecca Van Beneden, Professor of Biochemistry and Marine Sciences

ENDOCRINE DISRUPTION IN ATLANTIC SALMON (*Salmo salar*) EXPOSED TO PESTICIDES

By Benjamin W. Spaulding

Thesis Advisor: Dr. Terry Haines

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Master of Science
(in Zoology)
May, 2005

Since the early 1980's, the numbers of adult Atlantic salmon (*Salmo salar*) returning to Maine's rivers have been in a general decline. In addition, estimates of parr, freshwater smolt and emigrating smolt populations indicate low overwinter survival rates. Overall, these low numbers, along with several other factors, resulted in the Atlantic salmon in eight rivers in Maine being classified as a distinct population segment under the Endangered Species Act. Because many of the listed rivers are found near lowbush blueberry barrens, I investigated the endocrine disrupting potential and effects of selected blueberry pesticides on Atlantic salmon. An E-SCREEN assay was conducted to determine the relative estrogen mimicking properties, measured by a relative proliferative effect, of the most commonly used pesticides registered for use on lowbush blueberry.

Atlantic salmon (*Salmon salar*) pre-smolts of hatchery origin were subjected to pulsed exposures of a mixture of pesticides at environmentally

realistic concentrations. For each of the two years of this study, pre-smolts were subjected to a total of five weekly, 24 h pesticide exposures. In Year One, Velpar (hexazinone), Orbit (propiconazole), and Super BK (2,4 Dichlorophenoxy acetic acid) were tested. In Year Two, Orbit, Sinbar (terbacil), and Imidan (phosmet) were tested. To evaluate the effects of the pesticides on smoltification, the fish were periodically exposed to 24 h saltwater challenge tests (SWCT) to examine the osmoregulatory ability of the pre-smolts. Gill Na^+/K^+ -ATPase activity, plasma chloride concentration, hematocrit, vitellogenin presence, and plasma steroid concentrations (estrogen/androgen) of randomly selected smolts were measured after the pesticide exposures and saltwater challenges.

Control group Atlantic salmon pre-smolts did undergo smoltification as indicated by increased gill Na^+/K^+ -ATPase activity and low mortality rates in SWCT. Body length and weight of smolts was not affected by pesticide exposure in either year. Significantly lower gill Na^+/K^+ -ATPase activity was detected in smolts during Year Two only after the second SWCT. Plasma chloride levels were significantly different between control and exposed groups for both years, but overall values in each treatment remained in expected ranges for freshwater and saltwater portions of the study. Hematocrit values were within the normal range in Year One, but in Year Two exposed fish had significantly higher values than control fish after each pesticide exposure. Plasma steroid concentrations did not significantly differ between groups for either year. Therefore, in spite of multiple pulsed exposures to mixtures of blueberry pesticides calculated to be above expected concentrations found in the environment, the results do not

support the hypothesis that the observed overwinter mortality of smolts and reduced adult returns of Atlantic salmon are due to endocrine disruption by the pesticides utilized in this study.

ACKNOWLEDGEMENTS

I thank Dr. Terry Haines for serving as both my supervisor and advisor, and Dr. Rebecca Holberton and Dr. Rebecca Van Beneden for serving on my committee. This research and thesis would not have been possible without their assistance.

Funding for this project was provided by the U.S. Geological Survey and the U.S. Fish & Wildlife Service. I thank Wendy Morrill, Brian Perkins, Fred Trasko and the Green Lake National Fish Hatchery staff, David Yarborough, and the staff at the University of Maine Senator George J. Mitchell Center for Environmental Watershed Research for providing assistance with this study.

Special thanks go to my wife Amanda, family, and friends for their support and words of encouragement.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
INTRODUCTION.....	1
MATERIALS AND METHODS.....	6
Sediment collection.....	6
Pesticide exposures.....	9
<i>In vitro</i> experiments.....	9
Whole animal experiments.....	10
Salt water challenge tests.....	13
Sampling procedures.....	13
Analyses.....	15
Gill Na ⁺ /K ⁺ -ATPase activity.....	15
Plasma chloride concentration.....	16
Plasma vitellogenin concentration.....	16
Plasma steroid hormone concentration.....	17
RESULTS.....	20
Pesticide analysis.....	20
Physiology.....	24
Mortality.....	24

Length and weight.....	25
Gill Na ⁺ /K ⁺ -ATPase activity.....	30
Plasma chloride concentration.....	33
Hematocrit.....	33
Plasma vitellogenin concentration.....	38
Plasma steroid hormone concentration.....	38
DISCUSSION.....	44
Pesticide analysis.....	44
Physiology.....	45
CONCLUSIONS.....	52
REFERENCES.....	54
BIOGRAPHY OF THE AUTHOR.....	57

LIST OF TABLES

Table 1. Return of sea run adult Atlantic salmon to traps and weirs in 2003 as compared to the previous 23 years.....	2
Table 2. Sediment pesticide residue detection limits.....	21
Table 3. Year One Exposure tank pesticide concentration (ng/g).....	22
Table 4. Year Two Exposure tank pesticide concentration (ng/g).....	22
Table 5. Summary of the Relative Proliferative Effect (RPE) of compounds tested by E-SCREEN Assay.....	23
Table 6. Vitellogenin ELISA Results for Year One Smolts (2002).....	40

LIST OF FIGURES

Figure 1. Life Cycle of the Atlantic salmon (<i>Salmo salar</i>).....	3
Figure 2. Location of the Narraguagus River, Maine.....	7
Figure 3. Length of smolts over time for Year One.....	26
Figure 4. Length of smolts over time for Year Two.....	27
Figure 5. Weight of smolts over time for Year One.....	28
Figure 6. Weight of smolts over time for Year Two.....	29
Figure 7. Gill Na ⁺ /K ⁺ -ATPase activity of smolts over time for Year One.....	31
Figure 8. Gill Na ⁺ /K ⁺ -ATPase activity of smolts over time for Year Two.....	32
Figure 9. Plasma Chloride concentration of smolts over time for Year One.....	34
Figure 10. Plasma Chloride concentration of smolts over time for Year Two.....	35
Figure 11. Hematocrit Values of smolts over time for Year One.....	36
Figure 12. Hematocrit values of smolts over time for Year Two.....	37
Figure 13. Plasma Estradiol concentrations of smolts over time for Year One.....	41
Figure 14. Plasma Estradiol concentrations of smolts over time for Year Two.....	42
Figure 15. Plasma Androgen concentrations of smolts over time for Year One.....	43

INTRODUCTION

Atlantic salmon in eight rivers in Maine have been classified as a distinct population segment under the Endangered Species Act. Documented returns of sea run adult salmon to Maine (Table 1) show the decline of this species over the past 23 years. Previous research (Magee *et al.*, 2001) showed that smolts in one of these rivers (Naraguagus) had abnormally low gill Na^+/K^+ -ATPase activity and reduced survival rates in saltwater challenge tests (SWCT), demonstrating that they had impaired ability to osmoregulate in seawater. In addition, estimates of parr, smolt, and emigrating smolt populations indicate a low overwinter survival rate (FWS, 2000). The cause(s) of these low survival rates have not yet been determined.

The Atlantic salmon is an anadromous fish with a complex life history that includes several distinct life stages (Figure 1). Spawning occurs in November and the deposited eggs mature over the winter months. In early spring, the eggs hatch and the salmon begin their life in the freshwater environment. After spending approximately two years in their native stream or river, parr undergo the smoltification process to prepare for the marine environment (McCormick, 1987; Bjornsson, 1997). Studies have shown that salmon undergoing the parr-smolt transformation require specific conditions (especially with respect to water temperature and pH values) to complete the process (Kroglund, 1999). In addition, it has been shown that pre-smolts exposed to acidic water and aluminum in the freshwater environment were unable to survive the transition to

Table 1. Return of sea run adult Atlantic salmon to traps and weirs in 2003 as compared to the previous 23 years. Rivers in bold type are currently listed as Distinct Population Segment (DPS) rivers (Keliher, 2003 and USASAC Report, 1999).

Drainage	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990
Androscoggin				20	91	21	81	26	14	19	184
Dennys	190	126	38	28	68	20	15	1	9	12	32
Narraguagus	112	70	68	88	67	56	45	35	37	39	50
Penobscot	3244	3377	4130	946	1764	3343	4520	2441	2793	3047	3314
Pleasant	5	23	18	37	17	31	19	9		0	0
Saco						60	37	40	38	18	73
St. Croix		79	99	122	244	350	325	375	384	232	103
Union	239	295	149	146	40	82	67	62	47	30	21
TOTAL	3790	3970	4502	1387	2291	3963	5109	2989	3322	3397	3777

Drainage	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
Androscoggin	21	15	44	24	16	39	1	4	5	3	5	2	3
Dennys	7	5	13	6	5	10	0	1		2	17	2	9
Narraguagus	83	68	88	47	51	59	37	22	32	23	32	8	21
Penobscot	1732	2370	1750	1038	1332	2026	1355	1210	967	534	788	779	1112
Pleasant	0	0	0	2			1			3	11	0	2
Saco	4	0	58	23	34	51	28	28	66	49	69	47	39
St. Croix	185	1	103	83	46	132	28	41	13	20	20	20	15
Union	8	4	0			69	8	13	9	2	0	5	1
TOTAL	2040	2463	2056	1223	1484	2386	1458	1319	1092	636	942	863	1202

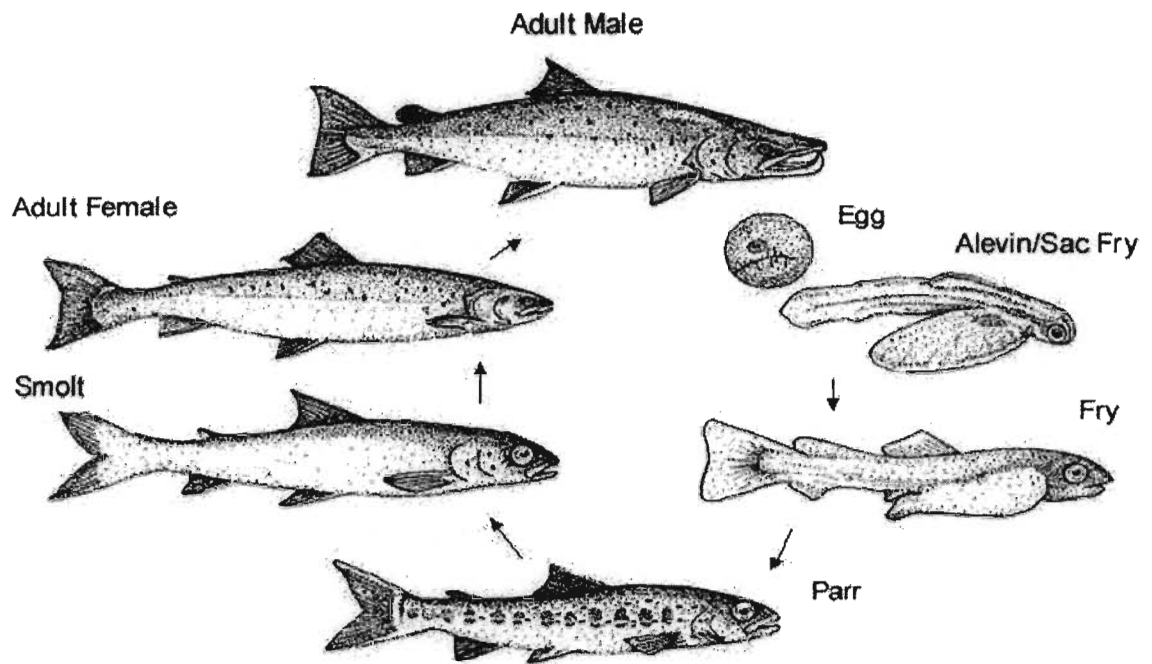


Figure 1. Life Cycle of the Atlantic salmon (*Salmo salar*). U.S. Fish and Wildlife Service.

a marine environment (Staurnes *et al.*, 1993; 1996). With a life history that spans multiple aquatic environments, there are many factors that could be contributing to the declining numbers of adult Atlantic salmon.

Many of the salmon rivers in Maine receive pesticide runoff from nearby blueberry barrens. Nineteen chemicals are registered for use on lowbush blueberries in Maine, including insecticides, herbicides, and fungicides (Dill *et al.*, 1998). Some of these are potent neurotoxins that interfere with olfaction in fish, causing impairment in homing ability and predator avoidance (Morin *et al.*, 1992; Moore *et al.*, 1996). Some may be hormone mimics that disrupt fish endocrine systems (Waring *et al.*, 1997; Moore *et al.*, 2003). The herbicide, Velpar (active ingredient hexazinone), has been detected in Atlantic salmon rivers year-round, and recently the organophosphate insecticide Imidan (active ingredient phosmet) was detected in both the Narraguagus and Pleasant rivers (Jackson *et al.*, 2003).

Hormone mimics, and their effects on the endocrine system, are a relatively new area of concern and research. Early studies examined their effects on estrogen receptors and binding activity (Lazier *et al.*, 1985). The effects of 17 β -estradiol injections on Atlantic salmon were tested by Madsen and Korsgaard (1989). Research on pesticides and their possible roles as endocrine disruptors have included both the pesticide DDT (Celius *et al.*, 1998) and the compound 4-nonylphenol (Fairchild *et al.*, 1999). These studies indicated that the major physiological parameters that may be affected by the presence of hormone mimics are gill Na⁺/K⁺-ATPase activity and relative gill Na⁺/K⁺-ATPase mRNA expression (Madsen *et al.*, 1997), plasma chloride levels, hematocrit,

vitellogenin induction (Tyler *et al.*, 1999), and plasma estradiol and androgen concentrations. All of these parameters are important in the smoltification process. Disruption of any part of this process by estrogen mimics may contribute to the decline in returning adult Atlantic salmon by reducing saltwater tolerance, and delaying smolt development and downstream migration (Madsen *et al.*, 2004).

I conducted this study in an effort to understand the extent that blueberry pesticides occur in Atlantic salmon rivers in Maine, their endocrine disrupting potential, and the possible role these contaminants might play in the smoltification of Atlantic salmon. The study consisted of two components. First, Narraguagus river sediment was collected and analyzed for the presence and identification of blueberry pesticides. Then various pesticides commonly used on lowbush blueberries were tested to determine their estrogenic potential. These results were used to shape the second component of the study, which was the testing of pre-smolts for the effects of pesticide exposure as they underwent smoltification. Fish were periodically subjected to saltwater challenge tests to determine the level of smoltification and effects of pesticides on osmoregulatory ability. Survival rates and several different physiological parameters were studied to assess the response of the fish to pesticide exposure. I hypothesized that exposure to a mixture of pesticides used in lowbush blueberry production would lead to a decrease in gill Na^+/K^+ -ATPase activity and reduced survival in the saltwater challenge tests.

MATERIALS AND METHODS

Sediment collection

In July of 2001, I sampled Narraguagus River sediment to determine if pesticides were present, and if so, in what quantity. The first sampling site was on the upper end of the Narraguagus River watershed, north of Route 9 at River km 49.15 (Figure 2). The second site was collected north of Cherryfield, Maine via Route 193 at River km 11.17. Access to the river was provided via the State of Maine Little Falls Research Station. The final site was located in the town of Cherryfield, Maine, adjacent to Route 193, at the Cherryfield dam (upstream of the boat launch) at River Km 2.00. At each site, I collected three sediment grabs (up to a 6" depth) from randomly selected areas with an Ekman grab sampler. The sediment was stored in 250 mL clear, glass, precleaned jars (I-Chem brand #V221-0250). All samples were held in a cooler on wet ice (approximately 4 hours) until returning to the University of Maine, where they were stored at 4°C until analyzed for pesticide residues.

Sediment samples were analyzed for pesticide residues at the University of Maine Food Chemical Safety Laboratory by Brian Perkins. Sediment samples were oven dried at 95°C and then underwent a microwave extraction procedure. Standard gel permeation chromatography (GPC) on an O.I. Analytical GPC AutoPrep 2000 was employed to remove high molecular weight interferences. The resulting samples were then analyzed for pesticide concentrations via gas

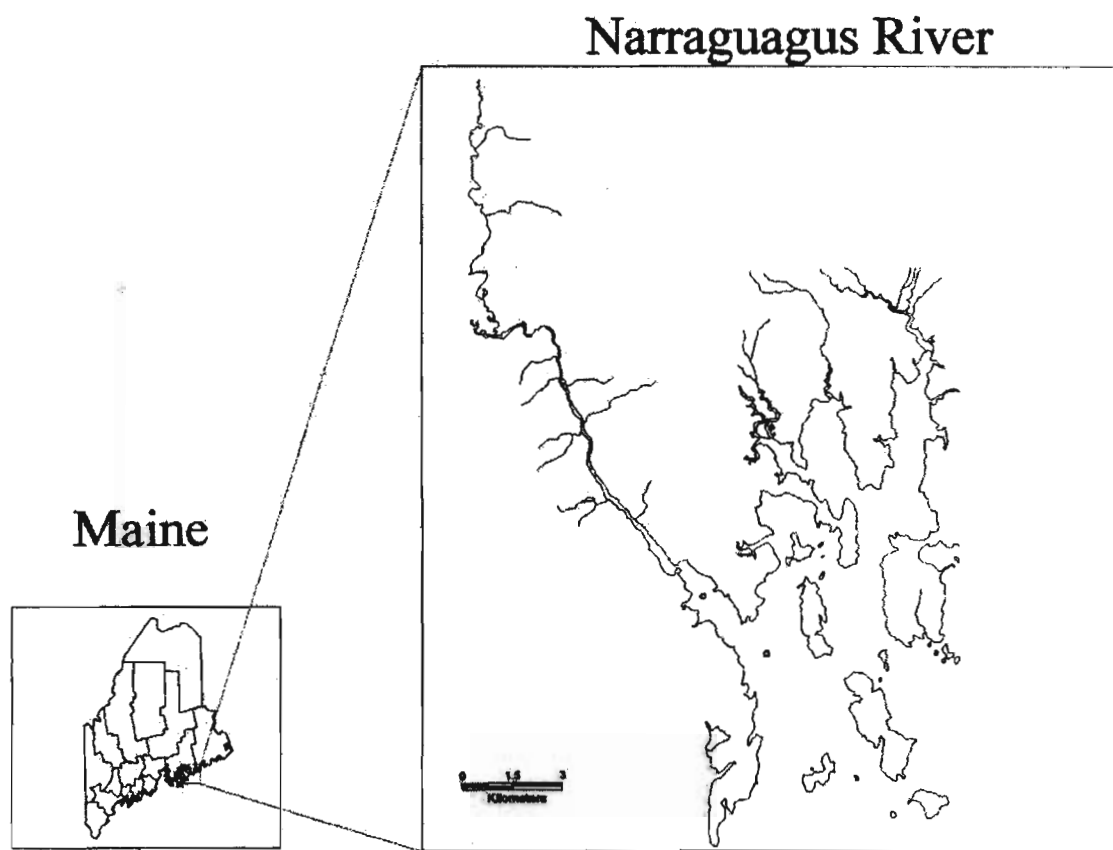


Figure 2. Location of the Narraguagus River, Maine.

chromatography mass spectrometer detector (GC-MSD) and high performance liquid chromatography with photodiode array detection (HPLC-PDA).

Sediment was analyzed using standard procedures modified by Perkins for the presence of the following: malathion, chlorothalonil, diuron, terbacil, propiconazole, methoxychlor, hexazinone, sethoxydim, clethodim, fluazifop-p-butyl. The first six pesticide residues were analyzed via GC-MSD and the last four by HPLC. Additionally, there was no further clean up of the sediment samples after the initial processing. The HPLC method encompassed the following parameters: The HPLC System consisted of a Hewlett Packard 1050 diode array detector, quaternary pump and auto sampler equipped with Chemstation software. The column was a Columbus, 5 μ particle sizes- 4.6 x 150 mm (Phenomenex, Inc., Torrance, CA). Mobile Phase was 60/40 (acetonitrile/water) with a flow rate of 1.0 mL/min and an injection volume of 50 μ L. Wavelengths monitored were 250 & 270 nm. A mixed standard (analytical grade pesticides standards, EPA Repository, Fort Mead, MD) was used to identify and quantify peaks of interest. The HPLC and the phenomenex spherisorb column were used for hexazinone in water analysis along with a hexazinone standard. The hexazinone analysis was accomplished under the same conditions as the above, with the following exceptions: The column was a Spherisorb with 5 μ particle size - 4.6 x 250 mm (Phenomenex, Inc., Torrance, CA), the mobile phase was 40:40:20 (acetonitrile:water:methanol) and the wavelength monitored was 247 nm.

Pesticide Exposures

In vitro experiments

All E-SCREEN assays were conducted at the University of Maine by Wendy Morrill. The E-SCREEN assay utilizes a human breast cancer (MCF-7) cell line (Soto *et al.*, 1995). The cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) in an atmosphere of 6.7% CO₂/93.3% air under saturating humidity, at 37°C. MCF-7 cells were plated into Falcon 12-well plates at a concentration of 30,000-40,000 cells/well. Test compounds were added directly to the medium, at three different concentrations (10 pM, 1 nM and 10 nM) and the cells were incubated at 37°C for 5 days. Scoring of the estrogenic effects of each xenobiotic was done by first measuring the proliferative effect (PE), which is the ratio between the highest cell yield counted with the test chemical to the yield of negative control cells (Soto *et al.*, 1995). PE was then used to determine relative proliferative effect (RPE), which is calculated as 100 times the ratio of the highest cell yield from the chemically-exposed cells to cells exposed to 17β-estradiol (Soto *et al.*, 1995).

Estradiol is assigned an RPE score of 100%, and all test xenobiotics were compared to estradiol. An RPE score of 100 to 50% indicates full to moderate xenoestrogenic activity, 50 to 20% indicates partial activity, and a score of <20% is accepted as having no activity. The experiments were repeated up to five

times per compound. Assay results that deviated more than two standard deviations from average were not used in the RPE calculations (Soto *et al.*, 1995). As of January 2004, all assays were counted using a Beckman Coulter Counter ViCell. Accuracy was verified using a hemocytometer.

Whole animal experiments

The second part of this study was conducted at the Green Lake National Fish Hatchery in Ellsworth, Maine. For the first year of this project, I used Velpar (hexazinone), Orbit (propiconazole), and Super BK32 (2,4-Dichlorophenoxy acetic acid) as the exposure pesticides because these compounds have been commonly used in blueberry production (note: Super BK32 is no longer used in blueberry fields), and had the highest estrogenic potential in preliminary E-Screen tests. For the second year, to test additional compounds used in blueberry production, I used Orbit, Sinbar (terbacil), and Imidan (phosmet). In order to detect potential endocrine disruption effects, pesticide mixture concentrations for each year were selected at a level higher than pesticides concentrations normally found in the environment and by the river sediment analysis results. Using published formulation data (MSDS), I calculated the final concentration for each pesticide mixture to be 1.0 mg/L of active ingredient of each pesticide. The exposure tank was filled to an approximate volume of 757 L (200 gal). Exposure tank water samples for analysis of pesticide concentrations

were collected approximately 15 minutes after the addition of the pesticide mixture, and again at approximately 24 h post mixing.

In the water analysis of the initial and 24 h post mixing values for propiconazole, 2,4-D, hexazinone, phosmet and terbacil, the following parameters were utilized using standard procedures modified by Perkins: for 2,4-D analysis (direct injection with no sample preparation) the HPLC system described above was used with a ProntoSIL 5 μ particle size 4.6 x150 mm column (MacMod), the mobile phase was 350 mL water, 200 mL methanol, 200 mL acetonitrile and 0.15 mL phosphoric acid. Flow rate was 1.0 mL/min with an injection volume of 50 μ L. The wavelengths monitored were 230 nm & 280 nm. For propiconazole, hexazinone, phosmet, and terbacil in water, sample preparation first consisted of a pass through a set of 50 mL methanol-activated, solid-phase extraction cartridges (Strata SDB-L, Styrene-divinylbenzene polymer [100 μ m, 260A] 500 mg/6mL [Phenomenex, Torrance, CA]). The cartridges were then dried under vacuum for 20 minutes and followed by an elution step using ethyl acetate (saving the first 1.0 mL for GC-MSD analysis). The GC-MSD (Agilent model 6890 GC with model 5973 MSD) conditions were as follows: the column was a J&W HP5 MS, 30 M x 0.25 mm ID with 0.25 μ m film thickness. Injection volume was 2 μ L at 250°C with helium as the carrier gas. Initial Flow was 1.2 mL/min at 12.5 psi with a pulse pressure of 25.0 psi for 1.5 minutes. Run time was seven minutes.

Penobscot strain Atlantic salmon one year pre-smolts were obtained from the Green Lake National Fish Hatchery, Ellsworth, Maine. The tanks (both

holding and pesticide exposure) used for this study were light blue, square, center drained, and fed with hatchery water, which is obtained from Green Lake. This water is moderately soft with low ion content (Magee, 1999).

The pesticide exposure tank and control tank drain was sealed. After the approximate necessary volume (for obtaining the correct pesticide concentration) of water was added to each tank, the external water supply was shut off. A submersible pump was placed into the center of the exposure and control tank and connected to the spray bar. These pumps recirculated the tank water by pumping the water through each tank's spray bar to provide aeration and water movement. To prevent an excessive increase in tank water temperature over the course of the 24 h exposure, a coiled hose was placed into each tank through which hatchery water was passed at a maximum rate to cool the tank water. To maintain a sufficient concentration of dissolved oxygen, an airstone was added to both the control and exposure tanks. These airstones were supplied by a 65 lb tank of oxygen for the duration of the exposure. Fish were transferred by dip net from their holding tank to their respective exposure tank. Control fish were transferred to a tank that had the same setup as the pesticide exposure tank, yet no pesticides were added. Observations of both exposed and control fish were made throughout the study and any abnormal behaviors were noted. After the 24 h exposure period, fish were transferred by dip net back to their respective holding tank. Water from the exposure tank was then pumped through a bed of activated carbon to remove any remaining pesticides.

Salt water challenge tests

Plastic 100 gal livestock watering tanks were utilized for each saltwater challenge. Each tank was filled with 75 gallons of hatchery water, and Instant Ocean (Aquatic Ecosystems, Apopka, FL) was added to bring the salt concentration up to the standard ocean range of 31 to 33 parts per thousand (ppt). A pump was installed on each tub to provide aeration. Coiled hoses with flowing hatchery water were placed into each tub to regulate water temperature. Finally, each tub was covered with netting to prevent smolts from jumping out.

For each of the two years, pre-smolts were subjected to a total of five, 24 h pesticide exposures, and three, 24 h saltwater challenge tests. Post pesticide exposure or SWCT, randomly selected fish were lethally sampled for analysis. In the first year (2002), the pesticide exposures occurred on March 14, 20, 28, April 3, and 11 with saltwater challenges occurring on March 24, April 2, and 10. In the second year (2003), the pesticide exposures occurred on March 6, 13, 20, 27, and April 3, with saltwater challenges occurring on March 18, 25, and April 1.

Sampling procedures

After each pesticide exposure and saltwater challenge, any mortality was recorded, along with observations of any abnormal behavior(s). Fish from each tank were sampled in groups of 5, for a total of 10 fish per treatment per sampling session. Smolts were anesthetized with buffered MS-222, at a

concentration sufficient to immobilize them within two minutes. Each smolt was then placed upside down on a wet sponge. Using a pair of fine surgical scissors, approximately 2-3 gill filament tips were cut from the second gill arch on the left side. The filaments were then placed into a vial containing ice cold SEI buffer (150mM sucrose, 10mM EDTA, 50 mM imidazole, pH 7.3; McCormick, 1993). These vials were then immediately placed into a liquid nitrogen storage vessel. The samples remained frozen in liquid nitrogen until transported to the University of Maine, where they were transferred to a -80°C ultra low freezer for storage.

To withdraw a blood sample from the caudal vein, a 3 mL pre-heparinized syringe equipped with a 25-gauge needle was used in conjunction with a pre-heparinized 1.5 mL microcentrifuge tube (MCT). The needle was inserted at the base of the caudal fin at an angle of approximately 45°. Once the vein was found, the plunger of the syringe was slowly withdrawn to obtain the blood sample. Once the rate of blood extraction had slowed, the needle was withdrawn, removed from the syringe and disposed of in an approved sharps container. The blood sample in the syringe was drained into an ice cold pre-heparinized MCT. From this tube, a subsample of blood was collected in two 100 µL hematocrit tubes and the ends of the tubes sealed with clay. The hematocrit tubes were then spun at 3,000 g for three minutes, read on a hematocrit tube reader, and the results recorded as percent of cells in the plasma.

The remaining blood samples in the 1.5 mL MCT were also centrifuged at 3,000 g for three minutes. Once spun 50 µL of plasma was removed, transferred

to a screw cap tube, and then placed into liquid nitrogen. In the first year, a total of four, 50 μ L plasma samples were collected from each fish to be analyzed for vitellogenin, androgen, estradiol, and plasma Cl^- . In the second year, a total of three, 75 μ L plasma samples were collected from each fish to be analyzed for vitellogenin, estradiol, and plasma Cl^- . After sampling, each fish was placed in a plastic bag and stored on ice. Upon return to the lab, each fish was removed from the plastic bag and length (nearest mm) and weight (nearest g) were recorded. Fish were then wrapped in aluminum foil and stored in a non-defrosting freezer.

Analyses

Gill Na^+/K^+ -ATPase activity

The determination of gill Na^+/K^+ -ATPase activity was made following the methods of McCormick (1993). I analyzed gill tissue samples within seven months post-collection date at the University of Maine. A microplate reader (VERSAmax, Molecular Devices Corporation, Sunnyvale, CA) was used in this analysis. All reagents were made at the time of analysis and all standard curves and quality control procedures were observed. For the accompanying standard protein analysis, a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was utilized following all of the manufacture's instructions. Both the ATPase and

protein assay were conducted at 25°C. The ATPase data analysis was done with SOFTmax PRO v3.1.2 software from Molecular Devices.

Plasma chloride concentration

Analysis for plasma chloride concentration was done at the University of Maine Senator George J. Mitchell Center for Environmental Watershed Research. Each plasma sample was allowed to thaw at room temperature. Once at room temperature, 20 µL of plasma was added to 10 mL of deionized H₂O. One plasma sample per sampling date was diluted a second time, as a replicate for quality control purposes, by adding 40 µL of plasma to 20 mL of deionized H₂O. Each dilution was analyzed by ion chromatography on a DX 500 Ion Chromatograph (IC) system (Dionex Corp., Sunnyvale, CA) following instrument manufacturer standard procedures.

Plasma vitellogenin concentration

Plasma samples from each year were analyzed for vitellogenin (VTG) by Wendy Morrill at the University of Maine (after Tyler *et al.*, 1999). The VTG assay was conducted at 4°C to prevent proteolysis. Phenyl methyl sulfonyl fluoride (PMSF) at 1mM final concentration was added to all samples, followed by 100 µL of primary Atlantic salmon Ab (AA-1 [Biosense, Norway], polyclonal Ab, diluted 1:1000 in 1% PBS). The plate was incubated overnight and washed

three times with TPBS (Tween, PBS). After washing, 200 μ L of 2% BSA in PBS was added to the wells and allowed to stand for two to four hours. The plates were washed again and then 100 μ L of positive and negative controls, from Biosense (diluted 1:3000 in 1% BSA in PBS) were added to the wells. The plates were incubated overnight and washed once again. 100 μ L of Ab (BN-5, monoclonal Ab [Biosense] diluted 1:1000 in 1% BSA in PBS) was added to each well and incubated four to 24 h. The plate was washed and 100 μ L of conjugated Ab (goat-anti mouse [BioRad #170-6516] diluted 1:3000 in 1% BSA in PBS) was added to each well. The plate was then incubated four to 24 h and washed five times with TPBS. After washing, 100 μ L of fresh developing solution (15 mg of 0.4 mg/mL o-phenylenediamine [Sigma P4664], 37.5 mL dH₂O, 15 μ L 30% H₂O₂) was added to each well. After incubating for 20-30 minutes, the reaction was stopped with 50 μ L of 4N H₂SO₄. Finally, each plate was read for optical density on a microplate reader at 492 nm.

Plasma steroid hormone concentration

The radioimmunoassay for plasma estrogen and testosterone concentration was based on the method of Wingfield (1992), as modified by Cash and Holberton (1999). For the first year of samples, both estradiol (E₂) and total androgen (T) concentrations were determined. Antibody used for these assays was obtained from Endocrine Sciences, Calabasas, CA. For the second year, only E₂ was analyzed, using antibody obtained from Lab Vision Corp.,

Fremont, CA. Sample volume was 50 μL of plasma, with a minimum volume of 10 μL if restricted by total plasma available. Each plasma sample (10-50 μL) was diluted with distilled water up to 200 μL . 20 μL of radiolabelled (^3H , approx. 2,000 cpm) estradiol or testosterone was added to each sample to determine the percentage recovered from the plasma sample by the extraction procedures used. Then, 4 mL of freshly distilled dichloromethane was added to each sample and allowed to stand for at least two hours. The resulting dichloromethane and extracted steroids layer was removed from each tube with a Pasteur pipette and placed into a new tube. The dichloromethane was evaporated under nitrogen gas in a water bath set at 37°C and the extract reconstituted with 550 μL of phosphate buffer (pH=7). Replicates of each sample were set up using 200 μL aliquots of the extract. To determine extraction efficiency (% recovery) of each sample, 100 μL of extract was added to glass scintillation vials, mixed with 4 mL of scintillation fluid (Packard Ultima Gold) and placed on the scintillation counter.

In addition to the extract replicates, a standard curve was set up for each run of samples. The standard curve consisted of 12 pairs of tubes. The first pair measured the total CPM expected from the amount of radiolabelled steroid to be added to each sample without the presence of charcoal (300 μL buffer+100 μL labeled steroid without charcoal), the second pair (300 μL buffer+100 μL labeled steroid) measured the non-specific (background) binding to components other than the antibody as the result of the charcoal and the third (200 μL buffer+100 μL labeled steroid+100 μL antiserum) was used to determine the maximum amount of binding that could occur by the radiolabelled steroid to the fixed

amount of antiserum that would be added to each unknown. The remaining nine tubes were a serial dilution of cold (commercially made, Sigma Chemical Co.) steroid (100 μ L buffer + 100 μ L cold steroid + 100 μ L labeled steroid + 100 μ L antiserum). Both the standard curve and extract replicates containing the 200 μ L aliquots of the sample extracts, 100 μ L each of the radiolabelled steroid and antiserum were well mixed and allowed to stand overnight in a refrigerator at $< 5^{\circ}\text{C}$.

Unbound radiolabelled and endogenous hormone was removed by incubation with 500 μ L dextran-coated charcoal (12 min at 4°C) followed by centrifugation at 2,000 rpm for 10 min at 4°C . The supernatant of each sample was then decanted into glass scintillation vials containing 4 mL of scintillation fluid. Each sample was counted on a Beckman LS6500 system for up to 10 min., or until 2% accuracy was reached. Samples were corrected for percent recovery. The assay sensitivity (minimum detectable dose) was 1.853 pg. A known standard sample was used to measure intra-assay variation, and a plasma pool sample was used to measure inter-assay variation.

RESULTS

Pesticide analysis

Sediment analysis for the three sampling sites on the Narraguagus River produced no measurable pesticide residues at the detection limits available (Table 2). Exposure water was analyzed for both years to determine the actual pesticide concentration (Tables 3 and 4). For Year One, mean concentration and range (initial/+24 h in ng/g) of each pesticide was: Orbit = 459/253 and 207-608/209-303, SuperBK32 = 343/283 and 183-461/246-332, Velpar = 91.4/89.0 and 71.6-103/70.7-104. For Year Two, mean concentration and range (initial/+24 h in ng/g) of each pesticide was: Orbit = 599/384 and 529-674/340-461, Imidan = 10.43/3.30 and 1.31-20.32/0.86-5.40, Sinbar = 464/442 and 403-515/374-492. For both years, actual pesticide concentration was lower than the calculated 1.0 mg/L of active ingredient.

The E-SCREEN assay was conducted on 22 individual compounds and two mixtures. Methoxychlor is the positive control for the assay. The relative proliferative effect (RPE) values (Ave \pm SD) for the individual pesticides used in Year One of this study were: Velpar = 15.5 % \pm 7.6, Orbit = 16.5 % \pm 6.2, SuperBK32 = 10.5 % \pm 4.9 and a mixture of all three (tested at a concentration of 0.5 ppm of each compound) was 15 % \pm 10 (Table 5). These RPE values, since they are below the value for Methoxychlor (18 % \pm 10.6), indicate that both the individual compounds and the mixture are negative for estrogen-like activity.

Table 2. Sediment pesticide residue detection limits.

Sample	LOQ (ng/g)	Method
Malathion	5	GC-MSD
Chlorothalonil	10	GC-MSD
Methoxychlor	10	GC-MSD
Diuron	10	GC-MSD
Terbacil	25	GC-MSD
Propiconazole	25	GC-MSD
Hexazinone	25	HPLC
Sethoxydim	25	HPLC
Clethodim	25	HPLC
Fluazifop-p-butyl	25	HPLC

LOQ = Limit of Quantitation

Table 3. Year One Exposure tank pesticide concentration (ng/g).

Sample	Orbit/ propiconazole	SuperBK32/ 2,4-D	Velpar/ hexazinone
Exp. 1, 0 h	207	288	85.2
Exp. 1, +24 h	236	260	88.3
Exp. 2, 0 h	547	183	98.1
Exp. 2, +24 h	239	293	93.1
Exp. 3, 0 h	428	431	71.6
Exp. 3, +24 h	209	332	70.7
Exp. 4, 0 h	503	354	99.2
Exp. 4, +24 h	303	246	104
Exp. 5, 0 h	608	461	103
Exp. 5, +24 h	278	*	*

* indicates analysis error-cannot quantify sample

Pesticide names are listed trade name/active ingredient

Table 4. Year Two Exposure tank pesticide concentration (ng/g):

Sample	Orbit/ propiconazole	Imidan/ phosmet	Sinbar/ terbacil
Exp. 1, 0 h	529	1.31	403
Exp. 1, +24 h	379	0.86	478
Exp. 2, 0 h	580	14.36	477
Exp. 2, +24 h	395	3.52	488
Exp. 3, 0 h	592	3.17	479
Exp. 3, +24 h	343	3.17	374
Exp. 4, 0 h	621	13.00	515
Exp. 4, +24 h	340	3.53	377
Exp. 5, 0 h	674	20.32	445
Exp. 5, +24 h	461	5.40	492

Pesticide names are listed trade name/active ingredient

Table 5. Summary of the Relative Proliferative Effect (RPE) of compounds tested by E-SCREEN Assay.

Test Compound	NAC	RPE (Ave + SD)
Velpar	4	15.5 \pm 7.6
Hexazinone	3	18 \pm 11
Orbit	4	16.5 \pm 6.2
Propiconazole	4	20.5 \pm 5.2
Super BK32	2	10.5 \pm 4.9
2,4 D acetic acid	4	13 \pm 5.8
Imidan 2.5EC	5	16.2 \pm 6
Phosmet	4	15 \pm 7
Sinbar	3	33 \pm 22.6
Terbacil	4	21 \pm 9.6
Mixture 1 (Velpar, Orbit, 2,4D)	3	15 \pm 10
Mixture 2 (Imidan 2.5EC, Sinbar, Orbit)	3	12 \pm 3.5
Clethodim	3	18 \pm 4
Diazinon	5	21 \pm 8.8
Diazinon 50W	3	21 \pm 10.6
Fluazifop p butyl	6	15.3 \pm 6.7
Methoxychlor (1 nM)	3	18 \pm 10.6
Sethoxydim	2	12.5 \pm 7.8
Poast	2	9
Benomyl	3	20.3 \pm 11
Benlate	3	10.3 \pm 6.4
Glyphosate	4	15.2 \pm 4
Round Up	5	17.4 \pm 9.9
Carbendazim	4	23 \pm 7.3

NAC = number of assays completed

Table reprinted with permission from Investigation of the Biological Effects of Agrochemicals by Rebecca Van Beneden and Wendy Morrill, 2004.

For Year Two, the RPE values were: Imidan-2.5EC = 16.2 % \pm 6, Orbit = 16.5 % \pm 6.2, Sinbar = 33 % \pm 22.6 and a mixture of all three (tested at a concentration of 0.5 ppm of each compound) = 12 % \pm 3.5. These RPE values indicate that Imidan, Orbit, and the mixture are negative, whereas Sinbar is positive for partial estrogen-like activity.

Physiology

Mortality

In Year One, at eight days post pesticide Exposure 1, there was one mortality in the control group of fish, and at 19 days post exposure, there was one mortality in the exposed group. The immediate causes of death are unknown. In Year Two, there were no mortalities during the experiments. The fish exposed to the pesticide mixture in Year Two exhibited mild lethargic behavior after the first exposure. However, after return to control water, the exposed fish seemed to partially recover as determined by a reduction in the display of lethargic behavior. As the pesticide exposures continued, the lethargic behavior continued in the exposed group. Other behaviors were noticed, especially after the fourth pesticide exposure. These included swimming higher in the water column, and brief losses of the ability to remain upright (with or without external stimulus).

Length and weight

In both years, fish length and weight had similar distributions between treatments (Figures 3 and 4). To determine significance in all figures, a general linear model (ANOVA) was used with an additional *post-hoc* Ryan-Einot-Gabriel-Welch test.

The average length value (\pm SD) for Year One was 19.4 ± 1.1 cm for exposed smolts and 19.2 ± 0.9 cm for SWCT smolts. The overall average body length of all fish used in Year One was 19.3 ± 1.2 cm. There were no significant differences in body length among groups used for any treatments except for post Exposure 3 smolts. At this exposure, the control group had a higher mean body length than the exposed group. In Year Two, the average length value (\pm SD) for exposed smolts was 19.3 ± 1.0 cm and 18.8 ± 1.0 cm for SWCT smolts. The overall mean length of Year Two smolts was 19.2 ± 1.0 cm. There were no significant differences between lengths of control and exposed smolts in Year Two.

Average weights (\pm SD) of smolts in Year One were 64.3 ± 11.6 g and 59.7 ± 11 g for exposed and SWCT fish, respectively. The overall average weight (\pm SD) of all fish in year one was 62.4 ± 12.9 g (Figure 5). Weights of control and exposed smolts were significantly different on two dates. The first was after Exposure 3 (control fish were significantly larger than exposed fish) and the second was after Exposure 4 (exposed fish had a larger range of values than control fish).

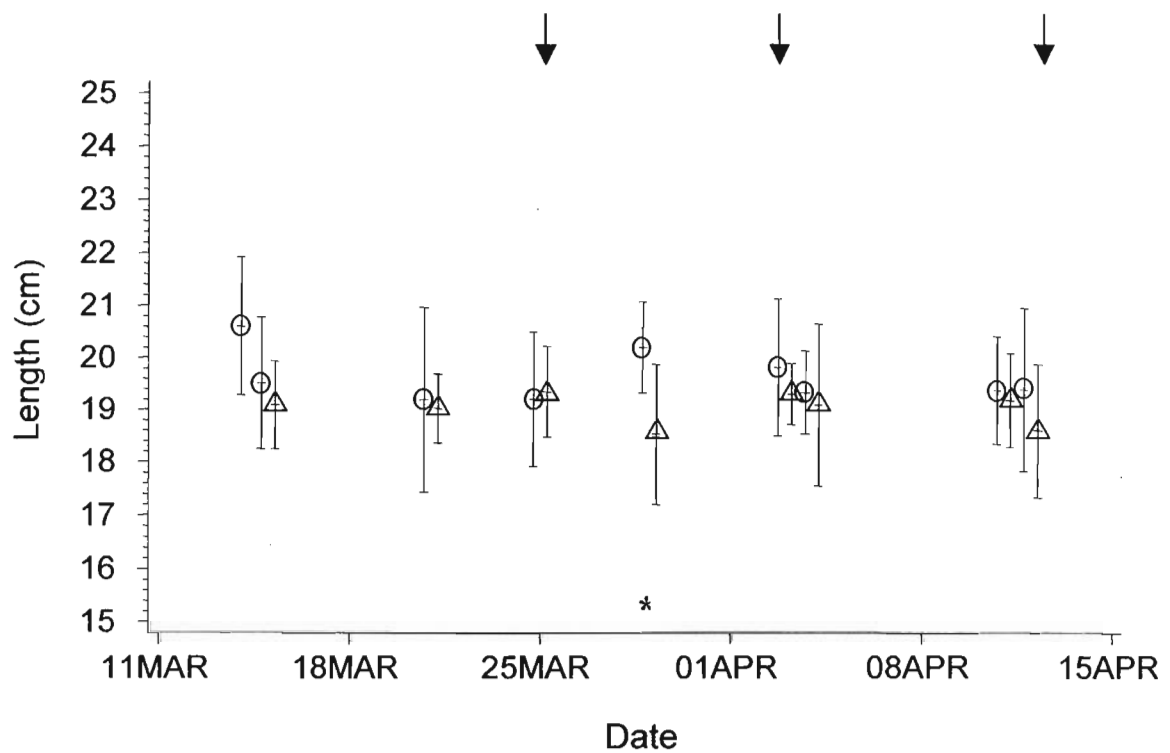


Figure 3. Length of smolts over time for Year One. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA and Ryan-Einot-Gabriel-Welch Test).

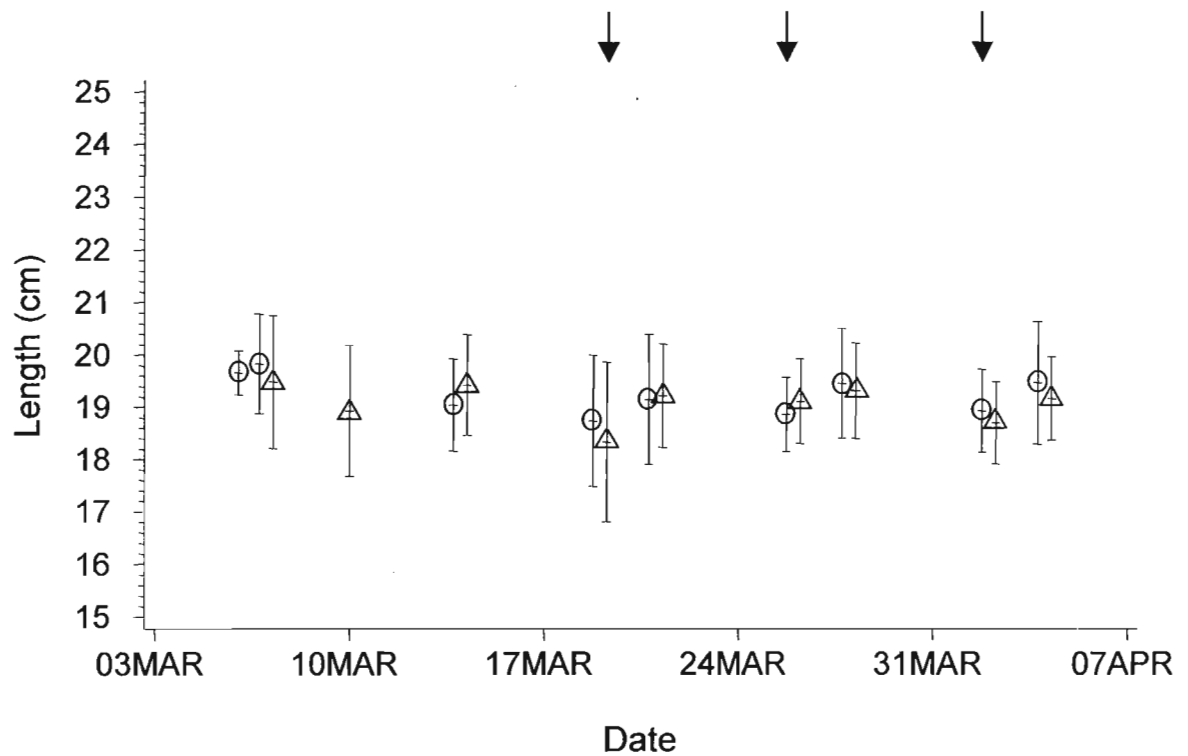


Figure 4. Length of smolts over time for Year Two.

Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each data point except SWCT 3 (n=9 [exp]). There were no significant differences at any time (ANOVA, $p > 0.05$).

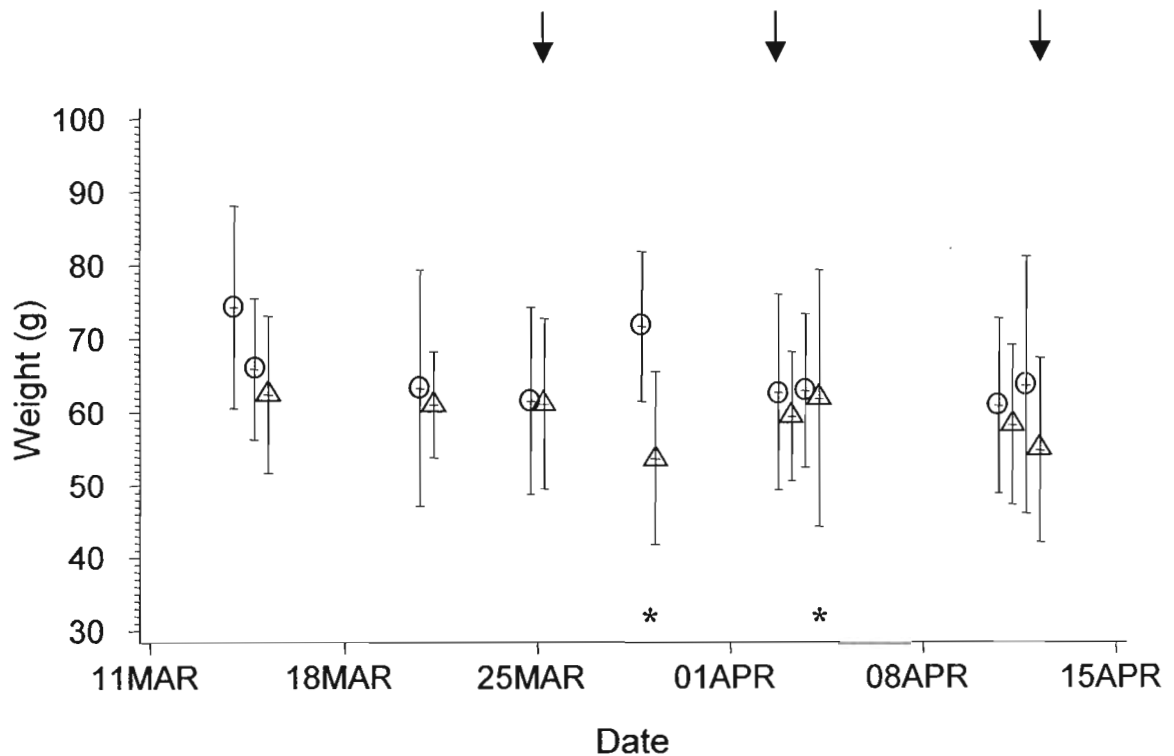


Figure 5. Weight of smolts over time for Year One.

Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

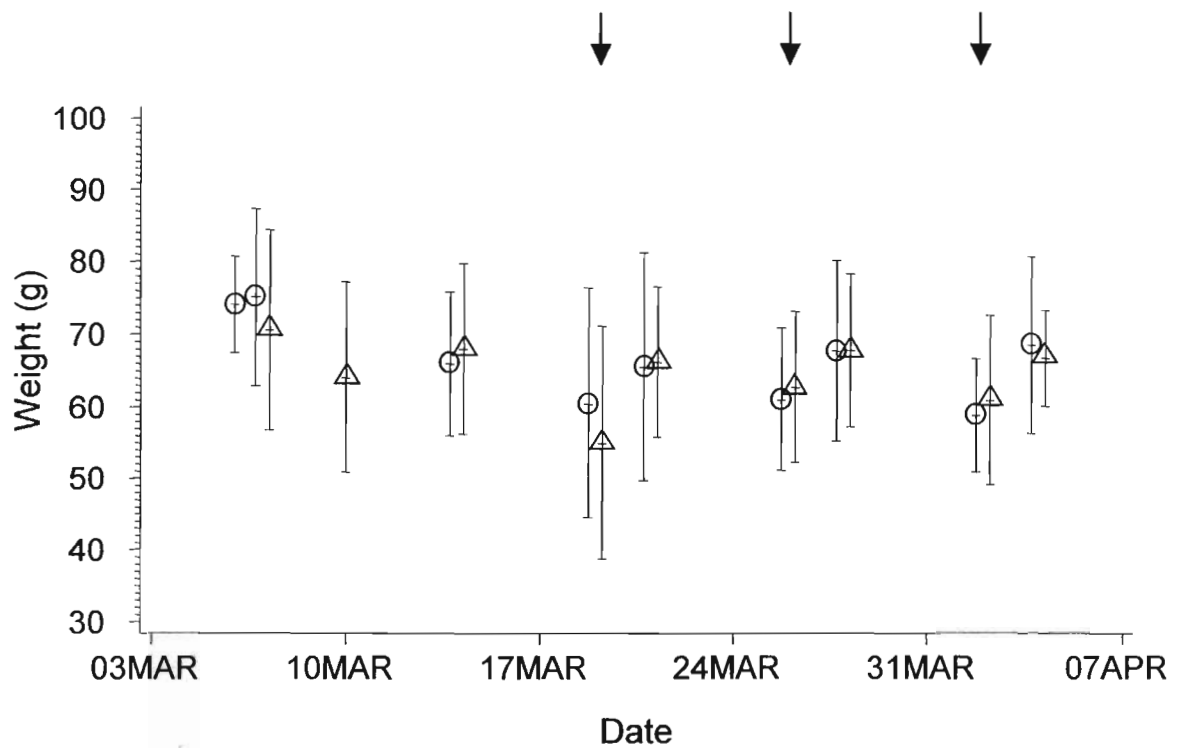


Figure 6. Weight of smolts over time for Year Two. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each sampling date.

For the second year, the average weight (\pm SD) was 68.3 ± 11.5 g and 59.7 ± 12.8 g for exposed smolts and SWCT smolts, respectively. The overall average body mass (\pm SD) was 65.4 ± 11.4 g (Figure 6). For this year, there were no significant differences in fish weight among treatments.

Gill Na^+/K^+ -ATPase activity

For both years of the study, initial ATPase values clustered around $3 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$ (Figures 7 and 8). In Year One, ATPase values increased until the March 28 sample, then declined slightly thereafter. There were no statistical differences between treatments except for the last two sampling dates, when exposed fish had significantly higher enzyme levels than control fish. In Year Two, noting the difference in dates between the two years of the study, ATPase levels did not increase over time, and were generally lower than during year one. Exposed fish had significantly lower ATPase levels than control fish at SWCT 2, Exposure 4 and Exposure 5.

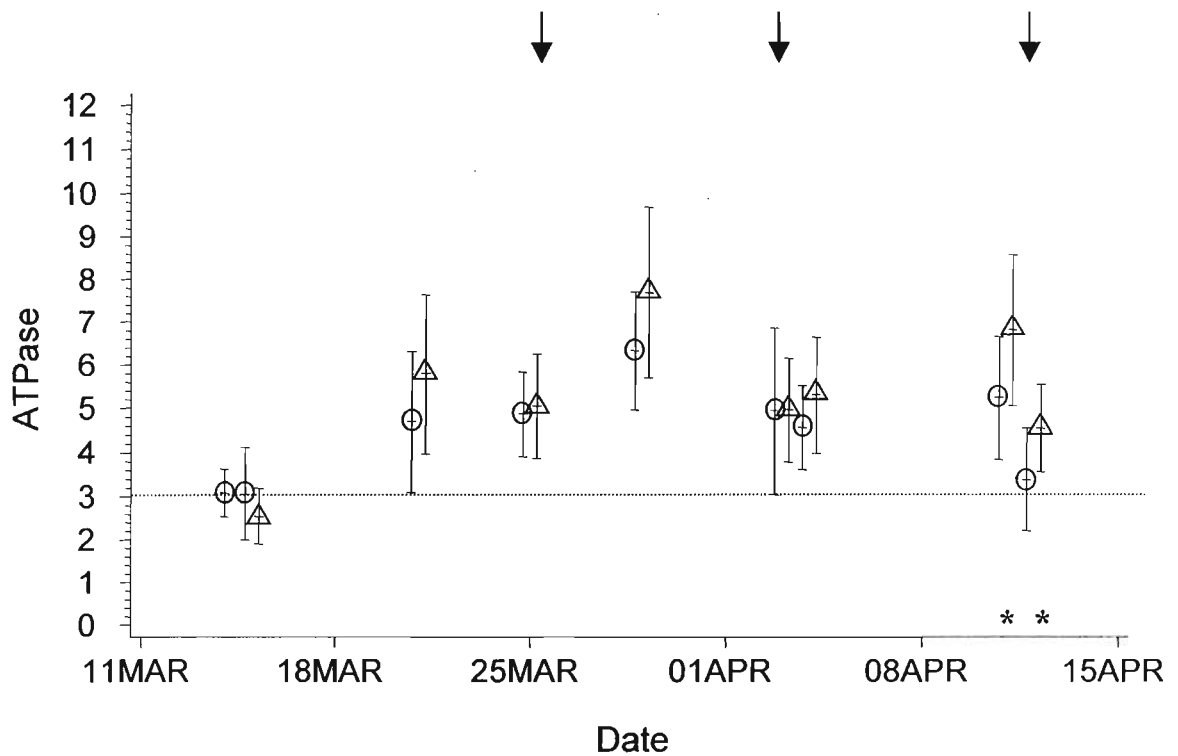


Figure 7. Gill Na^+/K^+ -ATPase activity of smolts over time for Year One. ATPase units = $\mu\text{mol ADP}/\text{mg protein}\cdot\text{hr}^{-1}$, Dashed line = minimum Na^+/K^+ -ATPase value for SW survival. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

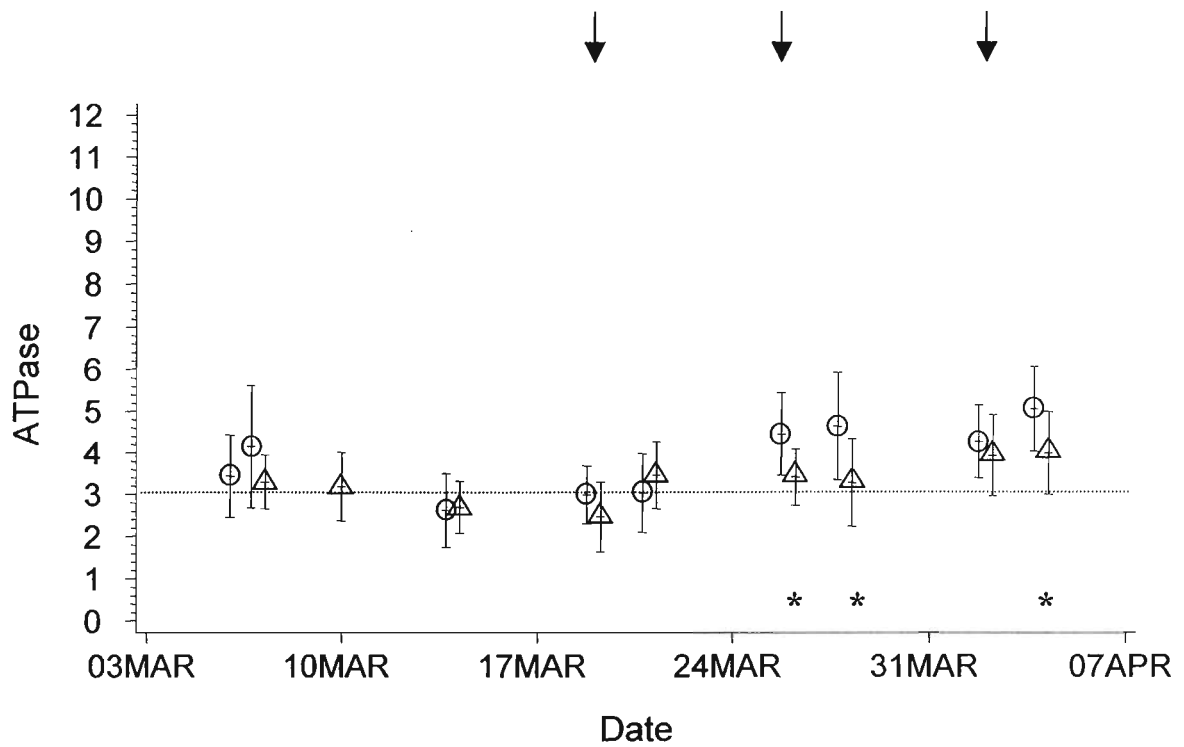


Figure 8. Gill Na^+/K^+ -ATPase activity of smolts over time for Year Two. ATPase units = $\mu\text{mol ADP}/\text{mg protein}\cdot\text{hr}^{-1}$, Dashed line = minimum Na^+/K^+ -ATPase value for SW survival. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 for each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

Plasma chloride concentration

In year one, plasma Cl^- of smolts in hatchery water varied between 130 and 140 meq/L and that of saltwater challenge fish varied between 140 and 160 meq/L (Figure 9). There were no significant differences between control and exposed fish except on March 28, when exposed fish had higher plasma Cl^- than control fish. Similar results were obtained in Year Two (Figure 10). There were significant differences between control and exposed fish in fresh water on March 7, 14, and 21. Exposed fish had higher plasma Cl^- on the first two dates, and lower on the third.

Hematocrit

In Year One, smolt hematocrit values generally were between 40 and 50% on all dates and exposure conditions, and were slightly higher than the normal range of 35-45% (Figure 11). There were no differences between exposed and control fish except on April 10, when exposed fish were significantly higher than control fish. The hematocrit reader used during this year was an older cylinder-type model that was difficult to read.

In Year Two, I used a new flat card-type tube reader to increase accuracy. Hematocrit values ranged between 35-45%, and were significantly higher in exposed fish than in control fish in fresh water on every test date (Figure 12). There were no differences between treatments in fish from any SWCT.

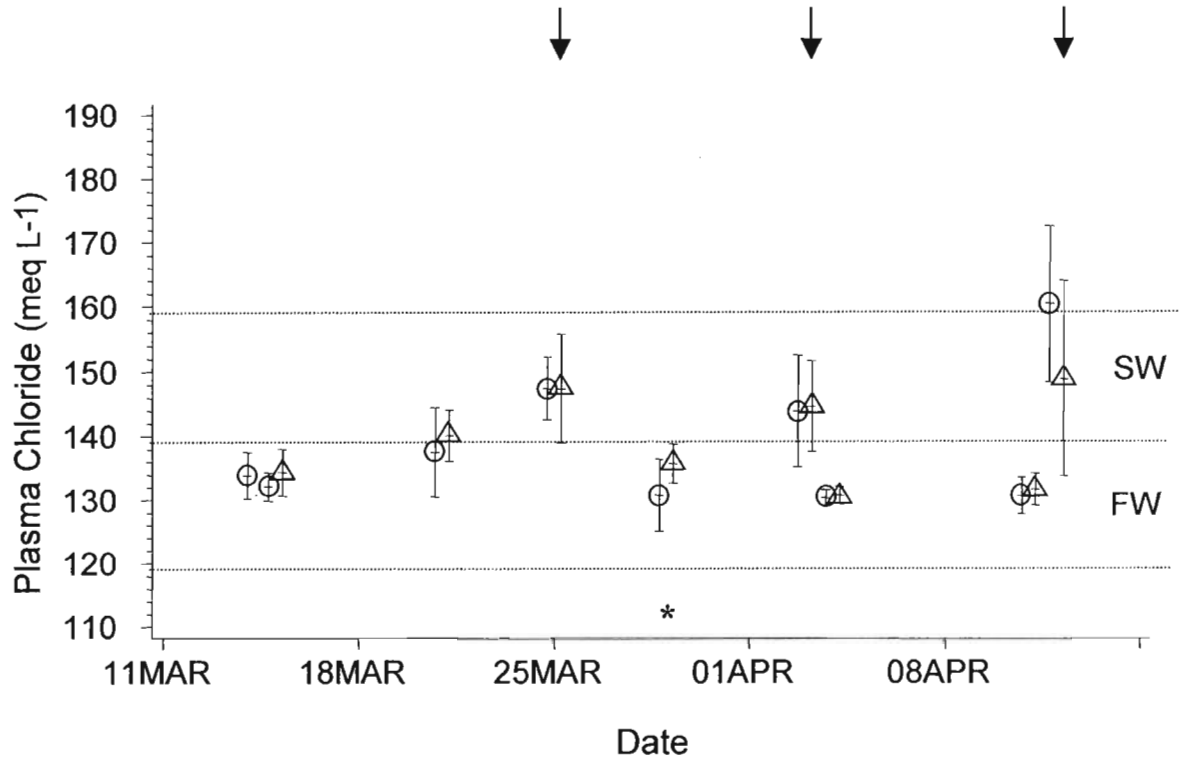


Figure 9. Plasma Chloride concentration of smolts over time for Year One. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. Dashed lines = ranges for freshwater (FW) and saltwater (SW) values. N = 10 for each data point except Control (n=9), Exposure 1 (n=8 [exp]), SWCT 1 (n=9 [exp]). * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

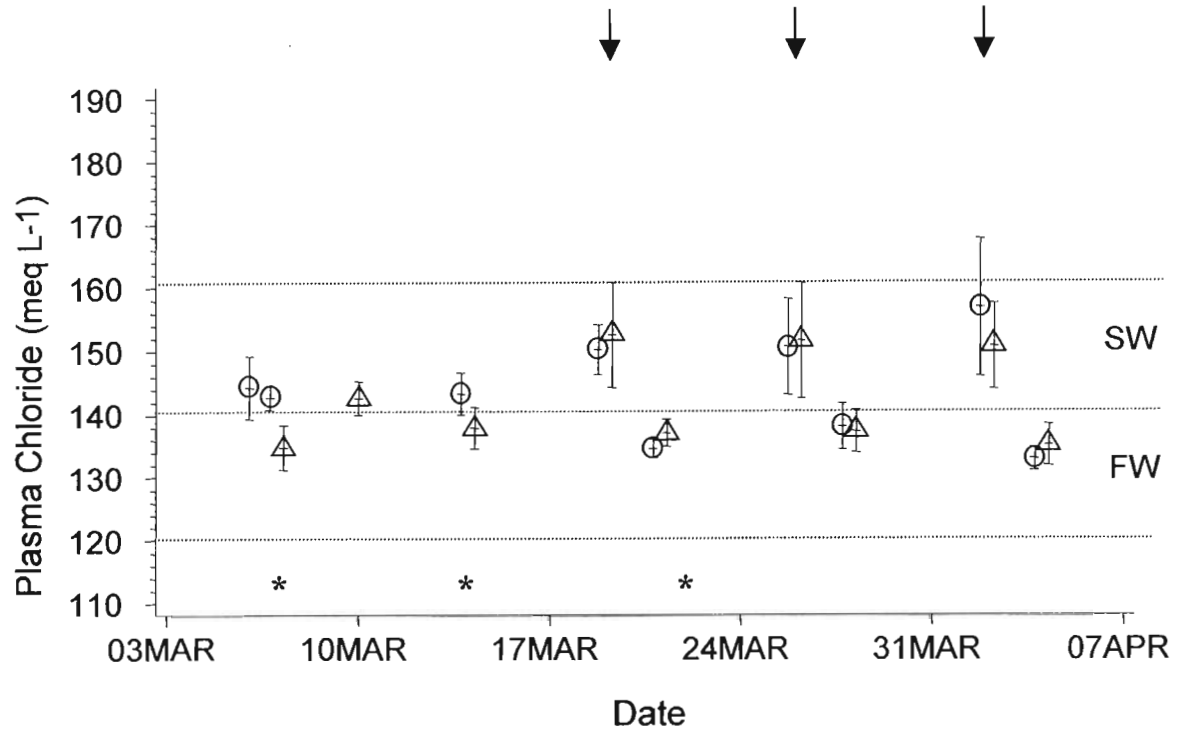


Figure 10. Plasma Chloride concentration of smolts over time for Year Two. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. Dashed lines = ranges for freshwater (FW) and saltwater (SW) values. N = 10 for each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

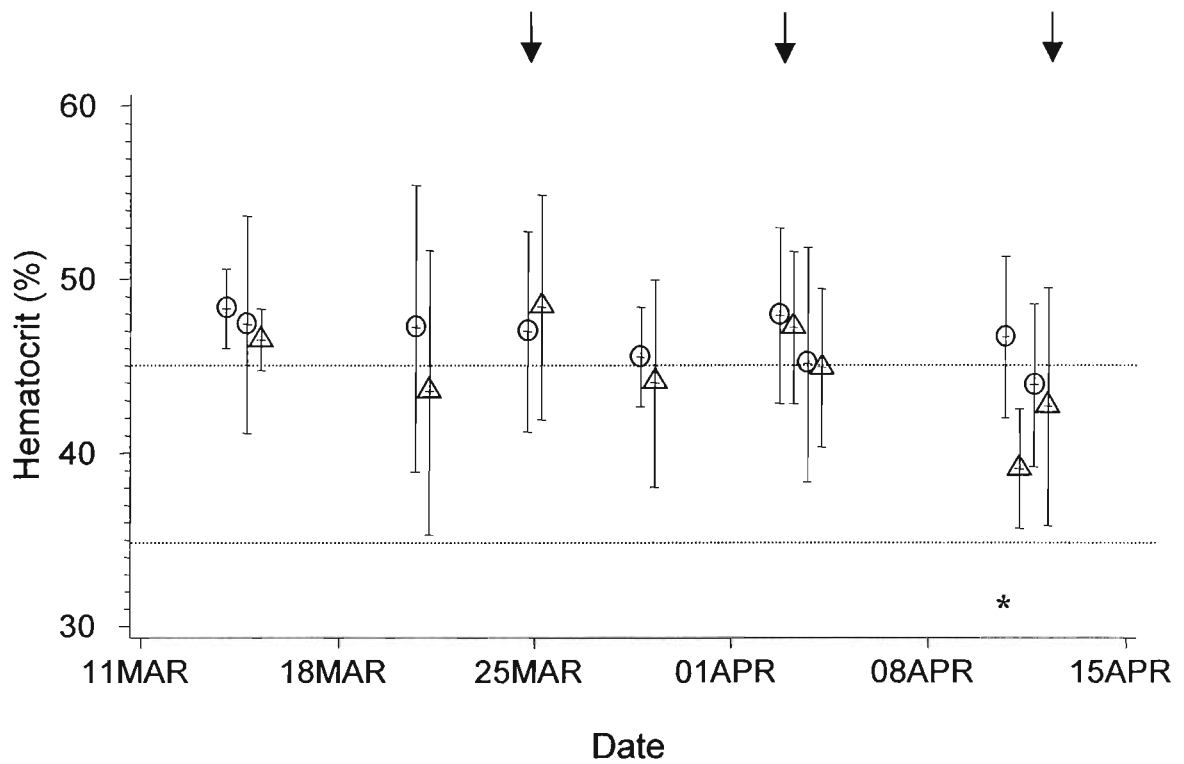


Figure 11. Hematocrit Values of smolts over time for Year One.

Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. Dashed lines = normal range for hematocrit values. N = 10 for each data point except Exposure 3 (n=8 [con], n=8 [exp]), SWCT 2 (n=9 [con]), Exposure 5 (n=9 [con]), SWCT 3 (n=9 [con], n=9 [exp]).

* = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

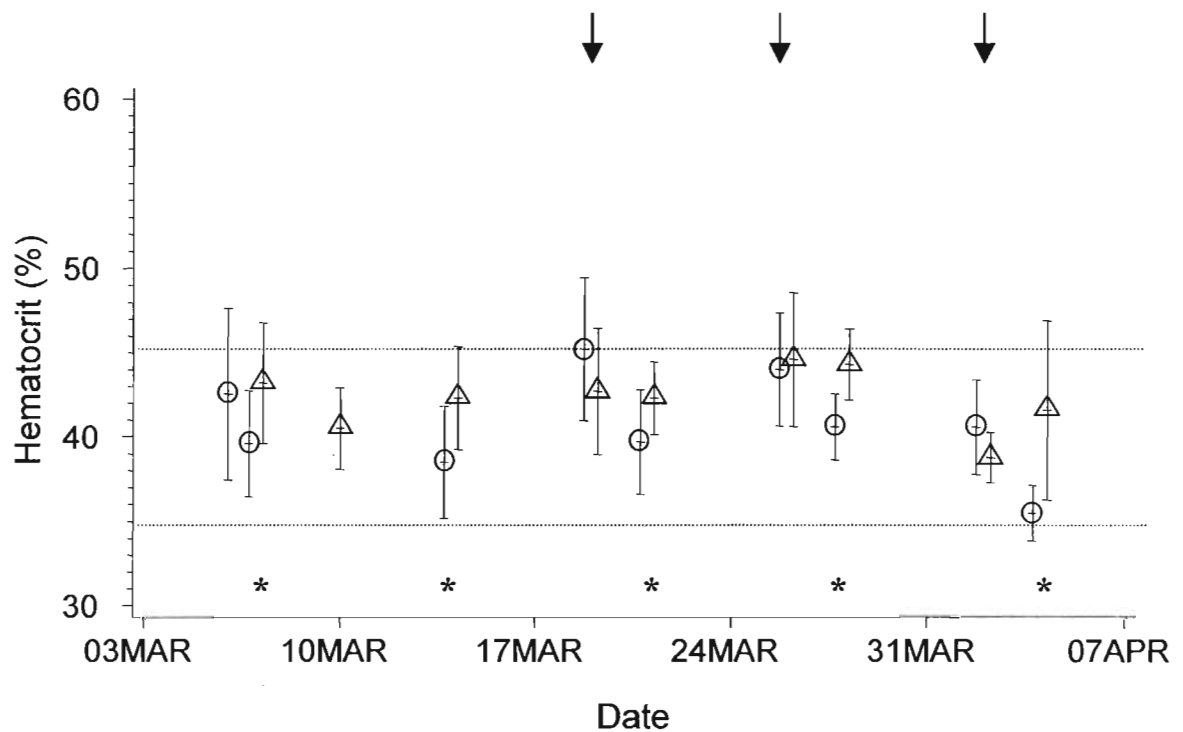


Figure 12. Hematocrit values of smolts over time for Year Two. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. Dashed lines = normal range for hematocrit values. N = 10 for each data point except Control (n=9). * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, Ryan-Einot-Gabriel-Welch Test).

Plasma vitellogenin concentration

Plasma samples from Year One were analyzed for vitellogenin expression (Table 6). Blank values ranged from 0.002-0.004 OD at 492 nm. Positive control (Atlantic salmon vitellogenin standard [Biosense]) values ranged from 0.322-0.495 OD at 492 nm with negative controls values of 0.003-0.012 OD at 492 nm. Experimental control samples had a similar range to the negative control samples. No detectable difference was found between exposure group and control group fish. The vitellogenin assay was not conducted during Year Two because of the negative results obtained in Year One.

Plasma steroid hormone concentration

In year one there were no significant differences in estrogen concentration of control and exposed smolts (Figure 13). Sampling date values for Exposure 1, Exposure 5 and SWCT 1 were omitted from the figure as they had values exceeding the expected range for estrogen concentration (Lazier *et al.*, 1985).

In Year Two, plasma estrogen was significantly lower in exposed smolts following Exposure 2 (Figure 14). During this one sampling date, the mean for the exposed group was significantly lower than the control group.

Total plasma androgen concentration was determined for Year One samples only. There were no significant differences between control and exposed smolts except for SWCT 2, where the control fish had lower androgen

levels than the exposed fish (Figure 15). During this one sampling date, the mean for the control group was significantly lower than the exposed group.

Table 6. Vitellogenin ELISA Results for Year One Smolts (2002).

All values are optical density (OD) at 492nm

Treatment	Positive control	Negative control	Row Average	Sample Values						
Control	0.357	0.004	0.005	0.001	0.006	0.005	0.002	0.011		
Control	0.495	0.012	0.016	0.004	0.009	0.013	0.032	0.023		
EXP 1 (con)	0.322	0.003	0.003	0.003	0.003	0.003	0.002	0.005		
EXP 1 (con)	0.495	0.012	0.023	0.011	0.023	0.021	0.029	0.030		
EXP 1 (exp)	0.495	0.012	0.033	0.015	0.050					
EXP 2 (con)	0.467	0.006	0.004	0.009	0.005	0.004	0.003	0.001		
SWCT1 (con)	0.467	0.006	0.000	0.003	-0.007	0.001	0.002	0.002		
EXP 3 (exp)	0.322	0.003	0.001	0.002	-0.007	-0.012	0.015	0.007		
SWCT2 (exp)	0.467	0.006	0.004	0.002	0.007	0.007	0.007	0.004	0.001	0.002
EXP 4 (exp)	0.357	0.004	0.004	0.005	0.005	0.006	0.002	0.004		
EXP 5 (exp)	0.322	0.003	-0.021	0.009	0.002	0.005	-0.010	-0.005	-0.051	-0.100
SWCT3 (exp)	0.357	0.004	0.005	0.008	0.003	0.001	0.003	0.010		

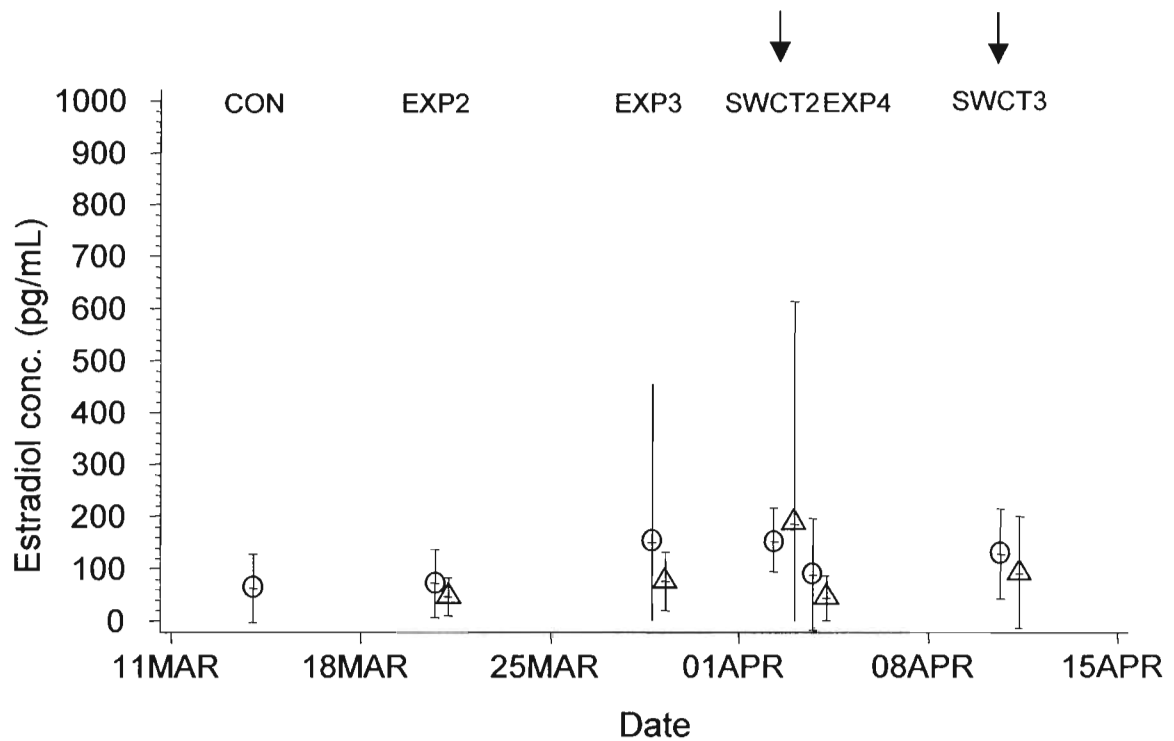


Figure 13. Plasma Estradiol concentrations of smolts over time for Year One. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = Control (n=9), Exposure 2 (n=7 [con], n=9 [exp]), Exposure 3 (n=9 [con], n=7 [exp]), SWCT 2 (n=8 [con], n=7 [exp]), Exposure 4 (n=9 [con], n=8 [exp]), and SWCT 3 (n=9 [con], n=9 [exp]). Note vertical axis scale when comparing Figures 13 and 14.

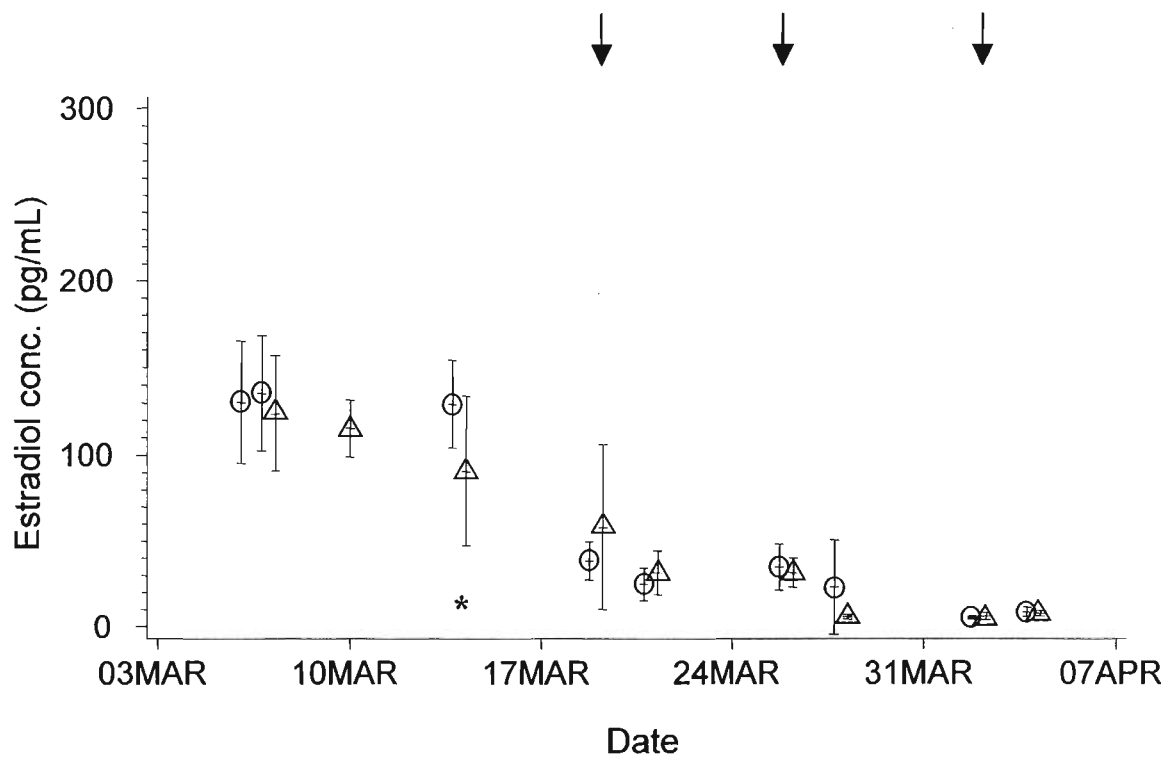


Figure 14. Plasma Estradiol concentrations of smolts over time for Year Two. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = 10 each data point. * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test). Note vertical axis scale when comparing Figures 13 and 14.

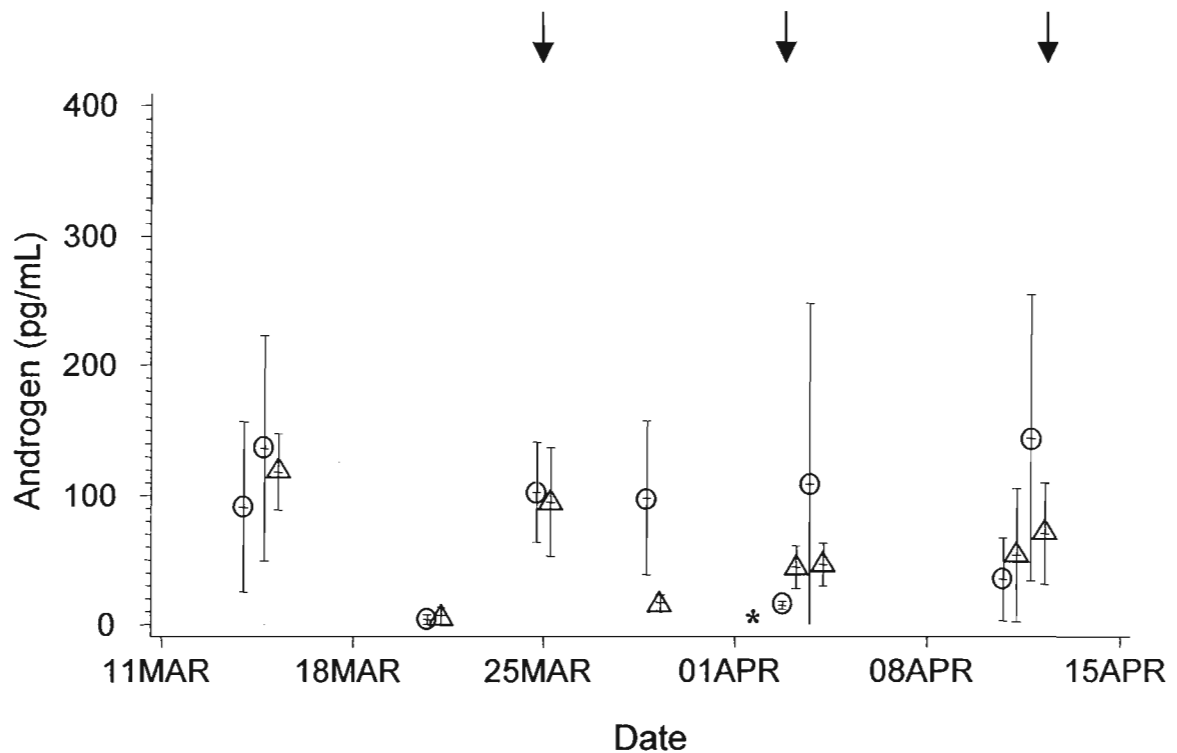


Figure 15. Plasma Androgen concentrations of smolts over time for Year One. Treatments = control (circle), exposed (triangle). Arrows = salt water challenge tests, Vertical bars = one standard deviation. N = Control (n=4), Exposure 1 (n=5 [con], n=5 [exp]), Exposure 2 (n=3 [con], n=3 [exp]), SWCT 1 (n=5 [con], n=5 [exp]), Exposure 3 (n=5 [con], n=3 [exp]), SWCT 2 (n=3 [con], n=4 [exp]), Exposure 4 (n=5 [con], n=5 [exp]), Exposure 5 (n=5 [con], n=5 [exp]), and SWCT 3 (n=4 [con], n=5 [exp]). * = significant difference between control and exposed treatments ($p < 0.05$, ANOVA, with Ryan-Einot-Gabriel-Welch Test).

DISCUSSION

Pesticide analysis

The sediment analysis indicated that there were no detectable pesticide residues in the Narraguagus River at our sampling sites. Residues may still be present in the river sediments, but at concentrations that would be below the standard detection limits of the assay. These results, combined with the drift studies of the Maine Board of Pesticide Control, suggest that pesticides are reaching the river but are not binding to organic matter. Variation in the concentrations of pesticide residues may still occur due to multiple factors that include method of application, time of application, pesticide stability and weather events. However, it can be concluded that Atlantic salmon pre-smolt and smolt exposure to pesticides (except Velpar) in the Narraguagus river region most likely occurs *via* pulse or short term *versus* a chronic long term exposure. Therefore, using 24 h pesticide and SWCT appears to be the best method for mimicking environmental exposures in a controlled laboratory setting.

Year One and Year Two exposure tank pesticide concentrations did follow the expected pattern of reduced pesticide concentration after each 24 h exposure period. This reduction in pesticide concentration may be due to the stability of each pesticide. During the 24 h pesticide exposures tank water was aerated, subjected to diffused sunlight (UV degradation), in contact with tank walls and equipment, and available to the fish. These factors, along with the chemical

properties of the pesticides, explain the reduction in pesticide concentrations observed for both years of the fish exposures.

The completion of the E-SCREEN analysis on a wide range of both individual and mixed pesticides provided the RPE value (and thus the potential endocrine disruption ability) of each compound. More recent research on endocrine disruptors in Atlantic salmon (Fairchild *et al.*, 1999; Madsen *et al.*, 1997, 2004) has focused on the effects of the compound 4-nonylphenol (4-NP). This compound, when administered by injection, disrupted or delayed smoltification. The experimental design for my study focused on pesticides that were known to be applied to blueberry barrens or detected in the Narraguagus river region. In summary, the RPE values of the pesticide mixtures used in this study were classified as negative for estrogenic activity. However, each mixture was selected not only due to the RPE value but in consideration of actual usage or previous detection in the study area.

Physiology

The pre-smolts used in the pesticide exposure portion of this study were larger than the fish used by Madsen *et al.* (1997, 2004). Their mean weight was approximately 23-24 g compared to the fish used in this study that had a mean weight of approximately 62-65 g. This size difference may explain the observed reduction (through a mass specific dose mechanism) in the physiological responses of the fish in this study. However, when compared to the referenced

studies by Madsen *et al.* (1997, 2004), the control and exposed fish had similar low mortality rates and the characteristic silvery smolt appearance. In addition, there were no major differences in average lengths and weights in either year of this study between control and exposed smolts.

One of the primary indicators of successful adaptation to a marine environment in the Atlantic salmon is gill Na^+/K^+ -ATPase levels. Several studies have shown that pre-smolts undergoing smoltification while exposed to 4-nonylphenol or 17β -estradiol have a significant reduction in gill Na^+/K^+ -ATPase activity and hypo-osmoregulatory performance (Madsen *et al.*, 1997). The general trend of the physiological effects of the pesticide mixtures, used in both years of this study, on gill Na^+/K^+ -ATPase activity did not exhibit the same reduction reported by Madsen *et al.* for other contaminants (1997). This may reflect the low RPE values of the pesticide mixtures used, or the route of fish exposure.

Year One smolts in this study during the time period of March 11-18 had gill Na^+/K^+ -ATPase levels of approximately $3 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$. These values continued to increase for both control and exposed fish until they reached the peak of enzyme activity of approximately $7 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$ around April 2. Enzyme activity then decreased after this time point until the end of the exposures. This trend in enzyme activity (along with the previously stated silvery appearance) suggests that the presmolts in both treatment groups in this year's exposures were indeed undergoing the smoltification process. There were few differences in ATPase activity between control and exposed fish in Year

One, and the one significant difference resulted when exposed fish had higher enzyme activity, the opposite of the expected response.

In Year Two, exposure to pesticides started approximately one week earlier than in Year One. This was to ensure that none of the pre-smolts had begun the smoltification process prior to pesticide exposure. The initial gill Na^+/K^+ -ATPase levels were approximately $4 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$. These values decreased slightly to below $3 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$ and then started to increase until peaking at approximately $4.5 \mu\text{mol ADP/mg protein}^{-1}/\text{hr}^{-1}$ around March 4. This peak was lower than the peak value observed for Year One. Thus smolts in Year Two may not have reached their maximum gill Na^+/K^+ -ATPase levels during the time frame of this year's sampling. This delay could be attributed to other non-experimentally controlled factors such as water temperature variations or even duration of light exposure inside the hatchery building.

In Year Two, exposed fish had significantly lower enzyme activity on three occasions, indicating that the chemicals used in this portion of the study may have been more estrogenically active than those used in Year One. It is also possible that the earlier onset of exposures in Year Two may have increased the effectiveness of the chemicals. However, all fish survived the saltwater challenges, indicating that they were able to osmoregulate successfully despite the reduced enzyme activities.

If exposure to pesticides truly impairs osmoregulatory ability, as reduced ATPase activity would indicate, then the exposed fish should have lower plasma

Cl⁻ than control fish in freshwater, and higher in saltwater. In both years of the study, however, exposed fish were able to maintain plasma Cl⁻ within the normal range of 120-140 meq L⁻¹, and saltwater exposed fish were able to maintain plasma Cl⁻ below 160 meq/L (Kroglund *et al.*, 2001). Although there were some differences in plasma Cl⁻ between control and exposed fish during both years of the study, these differences did not exceed the normal range, and thus for both years, smolts exposed to the pesticide mixtures were able to successfully complete the 24 h SWCT.

In contrast, previous work has indicated that transfer to saltwater induced significant dehydration (determined by change in muscle water content) and an increase in plasma Cl⁻ concentrations in Atlantic salmon smolts exposed by interperitoneal injection to 4-nonylphenol and 17 β -estradiol (Madsen *et al.*, 1997; Waring *et al.*, 2004). In this study, the results indicate that for most samplings and treatments, control and exposed fish had similar plasma Cl⁻ values. Thus fish exposed to either pesticide mixture did not exhibit significant increases in plasma Cl⁻ concentration and therefore may not be experiencing the same negative effects of pesticide exposure observed in previous studies.

Hematocrit is a measure of blood volume that is comprised of cells and other solid components. One way hematocrit can change is by the shrinking and swelling of cells. Values can fluctuate when there is osmotic stress and the smolt is unable to regulate these effects. In hyperosmotic environments without sufficient regulation, cells will shrink due to osmotic loss of water. In hyposmotic environments, cells will swell up due to osmotic water influx. However, other

factors are known to influence hematocrit values in Atlantic salmon. Previous work by Lacroix indicates that smolts emigrating from acidic rivers (with pH = 4.9 and 5.2) have hematocrit values above 50% with the smolt stage being more sensitive than the parr stage (1985). Also, the presence of labile, inorganic, monomeric aluminum at low pH significantly increased hematocrit levels in treated groups above control groups (Staurnes *et al.*, 1993). Finally, varied partial pressures (5.7 mmHg) of carbon dioxide were shown to increase hematocrit levels in Atlantic salmon (Fivelstad *et al.*, 2003). Therefore if the pesticide exposures utilized in this study were disrupting the smolts' ability to control Na^+/K^+ balance, hematocrit values would be expected to increase or deviate from the normal range depending on the type of treatment.

Hematocrit values for this study had large standard deviations in Year One. I believe this resulted from errors introduced while the tubes were read with the cylinder-type tube reader. Overall, the hematocrit values for Year One were slightly higher than the expected range. Year Two values analyzed with a new reader had lower variability. Readings were within the expected range of 35-45%, comparable to hematocrit values found in a previous study (Kroglund and Staurnes, 1999). Additionally, in Year Two, exposed fish had significantly higher hematocrit values than the control fish after each pesticide exposure yet no differences were detected after any of the SWCT. This would indicate that post-pesticide exposure in Year Two, smolts were not able to regulate osmotic stress at the same level as the control group. However, these significantly different means did fall in the established freshwater range. This suggests that although

the exposed smolts were exhibiting signs of minor osmotic stress, they were able to regulate their internal osmotic balance.

Induction and increased levels of plasma vitellogenin have been reported for teleosts exposed to potential endocrine disrupting compounds (Ankley *et al.*, 1997). In a recent study, plasma vitellogenin levels in smolts increased from control values of approximately 0.005 mg/mL to 4.0 mg/mL for 4-nonylphenol (120 µg/g body weight) and 11.0 mg/mL for E2 (2 µg/g body weight) exposed smolts (Madsen *et al.*, 2004). If exposure to the two pesticide mixtures was causing an induction or increased levels of plasma VTG levels, an ELISA assay should detect the presence of vitellogenin. Therefore, a vitellogenin ELISA assay was conducted on Year One plasma samples for both control and exposed fish. This assay provided optical densities of samples in comparison with positive and negative solutions. This was not a quantitative test as the concentration of vitellogenin could not be determined. The results of the assay showed that all samples tested were similar to the negative control values with no samples reaching the positive vitellogenin level.

Endocrine disrupting compounds can produce direct effects on steroidogenic enzymes or indirect modifications associated with altered feedback loops (Ankley *et al.*, 1997). One type of feedback and regulation that may occur with endocrine tissue and hormone production is negative feedback. In this study, the presence of xenoestrogens may act as a feedback signal to the original endocrine tissue. Previous research indicates that plasma concentrations of 17β-estradiol for Atlantic salmon range from 22 pg/mL to peak

values (during the vitellogenic phase) of 4-60 ng/mL (Lazier *et al.*, 1985). Thus, if one or more of the pesticides used in each year of this study were having direct or indirect effects on plasma concentrations of gonadal steroids, there should be significant differences between control and exposed treatment groups over time.

Year One estrogen and androgen concentrations do not exhibit any of the expected significant differences. Year Two estrogen concentrations for exposed fish decrease over time yet at the same rate as control fish. This decrease could be a natural fluctuation in circulating plasma steroid concentrations. Overall, the actual concentrations of plasma steroids are in the expected range (above 22 pg/mL but remaining an order of magnitude lower than the ng/mL range).

Therefore, as treated groups did not have either significantly higher or lower plasma steroid concentrations in Year One or Two than controls, the pesticide mixtures were not producing a detectable pattern of disruption on plasma concentrations of gonadal steroids. Several reasons for these results could be the relatively low RPE values of the pesticide mixtures (compared to 4-nonylphenol [RPE = 100 at 1 μ M]), the route of exposure (water *versus* injection), and exposure time (24 h *versus* chronic).

CONCLUSIONS

Based on the data and experimental design in the present study, it seems that common pesticides used on blueberry barrens in the Narraguagus river region in Maine are not present above detectable limits in river sediment. Atlantic salmon pre-smolts undergoing smoltification that were subjected to multiple, 24 h pesticide mixture exposures and saltwater challenge tests did not show significant indications of osmoregulatory or gonadal steroid disruption. Therefore, exposure of smoltifying Atlantic salmon to pulsed concentrations of blueberry pesticides does not support the hypothesis that the overwinter mortality of smolts and reduced adult returns is due to endocrine disruption by the pesticides utilized in this study.

Although I believe this study has provided some insight regarding the underlying reasons for low adult returns of sea run Atlantic salmon, additional research should be done on the issue of endocrine disruption. The majority of recent scientific experimentation on this subject has been conducted using injection of 4-nonylphenol and Atlantic salmon. New data provide evidence that there may be more than just a few endocrine disrupting compounds (EDC) present in the environment. RPE values can be calculated in a laboratory setting but practical application of this knowledge must be applied to the real life environment in which the animals of study reside. Future studies could incorporate a chronic exposure approach to pesticides found on a continuous basis in the environment. Researchers could also select compounds with high

RPE values to study the exact biochemical mechanisms and internal systems affected by exposure to these particular EDCs. As new analytical methods and assays are developed for detecting EDCs in the environment, they could be used to study the full effect of EDCs in relation to the Atlantic salmon and its complex life cycle. Therefore, as the effects of EDC become more prominent, research needs to be conducted to fully realize the potential hazards of the overuse of pesticides to both wildlife and humans.

REFERENCES

- Ankley, G. and 29 others. 1997. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environ. Tox. Chem. Ann. Rev.* 17: 68-87.
- Bjornsson, B.T. 1997. The biology of salmon growth hormone: from daylight to dominance. *Fish Physio. Biochem.* 17: 9-24.
- Cash, W.B. and Holberton, R.L. 1999. Effects of exogenous corticosterone on locomotor activity in the red-eared slider turtle, *Trachemys scripta elegans*. *J. Exp. Zool.* 284: 637-644.
- Celius, T. and Walther, B.T. 1998. Differential sensitivity of zonagenesis and vitellogenesis in Atlantic salmon (*Salmo salar* L) to DDT pesticides. *J. Exp. Zool.* 281: 346-353.
- Dill, J.F., Drummond, F.A. and Stubbs, C.S. 1998. Pesticide use on blueberry: a survey. Penn State Contract No. USDA-TPSU-UM-0051-1300. Univ. ME, Orono, ME.
- Fairchild, W., Swansberg, E.O., Arsenault, J.T. and Brown, S.B. 1999. Does an association between pesticide use and subsequent declines in catch of Atlantic salmon (*Salmo salar*) represent a case of endocrine disruption? *Environ. Health Perspect.* 107: 349-358.
- Fivelstad, S., Waagbø, R., Zeitz, S.F., Hosfeld, A.C.D., Olsen, A.B. and Stefansson, S. 2003. A major water quality problem in smolt farms: combined effects of carbon dioxide, reduced pH and aluminium on Atlantic salmon (*Salmo salar* L.) smolts: physiology and growth. *Aquaculture.* 215: 339-357.
- FWS (U.S. Fish and Wildlife Service). 2000. Endangered and threatened species; final endangered status for a distinct population segment of anadromous Atlantic salmon (*Salmo salar*) in the gulf of Maine. *Federal Register.* Vol. 65, No. 233. 69461. NOAA National Marine Fisheries Service - U.S. Fish and Wildlife Service. [Online]. Available: http://www.nero.noaa.gov/atsalmon/fr_fr.pdf [accessed October 28, 2004].
- Jackson, H.P. and Chizmas, J.S. 1999-2003. Drift study of two aerially applied blueberry pesticides. Maine Board of Pesticides Control report.
- Keliher, P. and Trial, J. 2003. 2003 Annual Report of the Maine Atlantic Salmon Commission.

- Kroglund, F. and Staurnes, M. 1999. Water quality requirements of smolting Atlantic salmon (*Salmo salar*) in limed acid rivers. *Can. J. Fish. Aquat. Sci.* 56: 2078-2086.
- Kroglund, F., Teien, H., Rosseland, B., Salbu, B. and Lucassen, E. 2001. Water quality dependent recovery from aluminum stress in Atlantic salmon smolt. *Water Air Soil Pollut.* 130: 911-916.
- Lacroix, G.L. 1985. Plasma ionic composition of the Atlantic salmon (*Salmo salar*), white sucker (*Catostomus commersoni*), and alewife (*Alosa pseudoharengus*) in some acidic rivers of Nova Scotia. *Can. J. Zool.* 63: 2254-2261.
- Lazier, C.B., Lonergan, K. and Mommsen, T.P. 1985. Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. *Gen. Comp. Endo.* 57: 234-245.
- Madsen, S.S. and Korsgaard, B. 1989. Time-course effects of repetitive oestradiol-17 β and thyroxine injections on the natural spring smolting of Atlantic salmon, *Salmo salar* L. *J. Fish Biol.* 35: 119-128.
- Madsen, S.S., Mathiesen, A.B. and Korsgaard, B. 1997. Effects of 17 β -estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). *Fish. Phys. Biochem.* 17: 303-312.
- Madsen, S.S., Skovbolling, S., Nielsen, C. and Korsgaard, B. 2004. 17- β Estradiol and 4-nonylphenol delay smolt development and downstream migration in Atlantic salmon, *Salmo salar*. *Aquatic Toxicology.* 68: 109-120.
- Magee, J.A. 1999. Effect of acid and aluminum on the physiology and migratory behavior of Atlantic salmon smolts in Maine. University of Maine Graduate School. Master's Thesis. 1:80.
- Magee, J., Haines, T., Kocik, J., Beland, K. and McCormick, S. 2001. Effects of acidity and aluminum on the physiology and migratory behavior of Atlantic salmon smolts in Maine, USA. *Water Air Soil Pollut.* 130: 881-886.
- McCormick, S.D. and Saunders, R.L. 1987. Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth, and metabolism. *Am. Fish. Soc. Symp.* 1: 211-229.
- McCormick, S.D. 1993. Methods for nonlethal gill biopsy and measurement of Na⁺, K⁺ -ATPase activity. *Can. J. Fish. Aquat. Sci.* 50: 656-658.
- Moore, A. and Waring, C.P. 1996. Sublethal effects of the pesticide Diazinon on olfactory function in mature male Atlantic salmon parr. *J. Fish Biol.* 48: 758-775.

Moore, A., Scott, A.P., Lower, N., Katsiadaki, I. and Greenwood, L. 2003. The effects of 4-nonylphenol and atrazine on Atlantic salmon (*Salmon salar* L) Smolts. *Aquaculture*. 222: 253-263.

Morin, P.-P. and Døving, K.B. 1992. Changes in the olfactory function of Atlantic salmon, *Salmo salar*, in the course of smoltification. *Can. J. Fish. Aquat. Sci.* 49: 1704-1713.

Soto, A.M., Sonnenshein, C., Chung, K.L., Fernandez, M.F., Olea, N. and Serrano, F.O. 1995. The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ. Health Perspect.* 103: 113-122.

Staurnes, M., Blix, P. and Reite, O.B. 1993. Effects of acid water and aluminum on parr-smolt transformation and seawater tolerance in Atlantic salmon, *Salmo salar*. *Can. J. Fish. Aquat. Sci.* 50: 1816-1827.

Staurnes, M., Hansen, L.P., Fugelli, K. and Haraldstad, O. 1996. Short-term exposure to acid waters impairs osmoregulation, seawater tolerance, and subsequent marine survival of smolts of Atlantic salmon (*Salmo salar* L.). *Can. J. Fish. Aquat. Sci.* 53: 1695-1704.

Tyler, C.R., van Aerle, R., Hutchinson, T.H. and Maddix, S. 1999. An *in vivo* testing system for endocrine disruption in fish early life stages using induction of vitellogenin. *Environ. Tox. Chem.* 18: 337-347.

USASAC (U.S. Atlantic Salmon Assessment Committee). 1999. Annual Report of the U.S. Atlantic Salmon Assessment Committee. Report No. 11-1998 Activities. Prepared for the U.S. Section to NASCO, by USASAC, Gloucester, MA. March 1-4, 1999.

Waring, C.P. and Moore, A. 1997. Sublethal effects of a carbamate pesticide on pheromonal mediated endocrine function in mature male Atlantic salmon (*Salmon salar* L.) parr. *Fish Physio. Biochem.* 17: 203-211.

Waring, C.P. and Moore, A. 2004. The effect of atrazine on Atlantic salmon (*Salmo salar*) smolts in freshwater and after sea water transfer. *Aquat. Tox.* 66: 93-104.

Wingfield, J.C., Vleck, C.M. and Moore, M.C. 1992. Seasonal changes in the adrenocortical response to stress in birds of the Sonoran desert. *J. Exp. Zool.* 264: 419-428.

BIOGRAPHY OF THE AUTHOR

Benjamin W. Spaulding is a native of the Bangor, Maine area. He graduated from Hermon High School in 1993. In the fall of that year, he began his college studies in the area of Biology at the University of Maine. After his sophomore year, he changed his degree to Biochemistry. He graduated with a B.S. in Biochemistry and minors in Biology, Microbiology, Molecular & Cellular Biology and Zoology in May, 1997.

In the fall of 1998, he started his professional career in the sciences under the supervision of Dr. Terry Haines at the University of Maine. For the next three years, he worked on and completed the Alkalinity Enhancement Project at the Craig Brook National Fish Hatchery, East Orland, Maine. Once completed, he began work on both his graduate degree and the project that would serve as the basis for his Master's degree: endocrine disruption of Atlantic salmon exposed to pesticides. He is a candidate for the Master of Science degree in Zoology from The University of Maine in May, 2005.